Université de Montréal

Systemic sclerosis immunoglobulin induces growth and a pro-fibrotic state in vascular smooth muscle cells through the epidermal growth factor receptor

par

Monique Arts

Faculté de pharmacie

Mémoire présenté en vue de l'obtention du grade de MSc en

Sciences Pharmaceutique option Pharmacologie

Août 2018

Résumé

La sclérose systémique (SSc) est une maladie auto-immune qui se caractérise par la dysfonction immunitaire, la fibrose et les vasculopathies. Il a été suggéré que les autoanticorps des patients atteints de la SSc peuvent induire la différentiation des fibroblasts en myofibroblasts via l'activation du récepteur du facteur de croissance dérivé des plaquettes (RPDGF). Notre étude a pour but de caractériser les effets des IgG des patients atteints de la SSc sur les cellules musculaires lisses vasculaires (CMLV) et de déterminer s'il est possible de détecter la présence d'autoanticorps dirigés contre le RPDGF, et s'ils induisent une réponse profibrotique chez les CMLV primaires cultivées.

Des CMLV ont été exposées à différentes fractions d'IgG purifiées du sérum des patients atteints de la SSc (IgG SSc) et d'individus non atteints (IgG témoins). La phosphorylation des protéines kinases ERK1/2 et AKT, la prolifération cellulaire, la synthèse protéique et l'expression de gènes profibrotiques ont été étudiées chez les CMLV stimulées, de même que le potentiel des IgG d'immunoprécipiter le RPDGF des lysats de CMLV.

L'activité stimulatrice des IgG SSc était plus grande que chez les IgG contrôles (p<0,05). Puis, les IgG SSc ont provoqué une immunoprécipitation plus importante du RPDGF que les IgG contrôles. Il était intéressant de constater que l'activation d'évènements de signalisation en aval du RPDGF, comme la phosphorylation d'Akt et d'ERK1/2, étaient indépendante de l'activité du RPDGF, mais nécessitaient la fonctionnalité du REGF. La stimulation des CMLV avec des IgG SSc, comparé aux IgG témoins, a induit une augmentation de la synthèse protéique (p<0,001) ainsi que la modulation pro-fibrotiques de certains gènes (*Tgfb1* +200%; *Tgfb2* -23%; p<0,001).

Comparé aux IgG contrôles, les IgG SSc avaient un index de stimulation plus élevé chez les CMLV. Même si les IgG SSc interagissaient avec le RPDGF, la signalisation passe par le REGF chez les CMLVs. Par conséquent, nos travaux soutiennent un modèle de transactivation du REGF par des autoanticorps anti-RPDGF provenant des patients atteints de la SSc et suggèrent que les inhibiteurs du REGF devraient être utilisés dans les études futures visant à identifier de cibles thérapeutiques pour la SSc.

Mots-clés : sclérose systémique, sclérodermie, autoanticorps, PDGFR, EGFR, cellules musculaires lisses vasculaires, inhibiteurs des protéines kinases, transactivation des récepteurs

Abstract

Systemic sclerosis (SSc) is an autoimmune disease characterized by the presence of autoantibodies, fibrosis and vasculopathy. It has been suggested that autoantibodies in systemic sclerosis may induce the differentiation of cultured fibroblasts into myofibroblasts through platelet-derived growth factor receptor (PDGFR) activation. The present study aims to characterize the effects of SSc IgG on vascular smooth muscle cells (VSMCs) and to determine if stimulatory autoantibodies directed to the PDGFR can be detected, and whether they induce a profibrotic response in primary cultured VSMCs.

Cultured VSMCs were exposed to IgG fractions purified from SSc-patient or control sera. VSMC responses were then analyzed for ERK1/2 and Akt phosphorylation, cellular proliferation, protein synthesis, and pro-fibrotic changes in mRNA expression. The capacity of the IgG fractions to immunoprecipitate the PDGFR from VSMC lysates was also tested.

Stimulatory activity in IgG fractions was more prevalent and intense in the SSc samples than in the controls ($p<0.05$), and SSc IgG immunoprecipitated the PDGFR with greater avidity than control IgG. Interestingly, activation of downstream signaling events (Akt, ERK1/2) was independent of PDGFR activity, but required functional epidermal growth factor receptor (EGFR). We also detected increased protein synthesis ($p<0.001$) and pro-fibrotic changes in gene expression (*Tgfb1* +200%; *Tgfb2* -23%; $p<0.001$) in VSMCs treated with SSc IgG.

When compared to control IgG, SSc IgG has a higher stimulation index in VSMCs. Although SSc IgG interact with the PDGFR, the observed remodeling signaling events occur through the EGFR in VSMC. Our data thus favour a model of transactivation of the EGFR by

SSc-derived PDGFR autoantibodies and suggest the use of EGFR inhibitors in future target identification studies in the field of SSc.

Key words: Systemic sclerosis, scleroderma, vascular smooth muscle cells, epidermal growth factor receptor, EGFR, receptor transactivation, autoantibodies, platelet-derived growth factor receptor, PDGFR, protein kinase inhibitors

Table of Contents

List of Figures

Chapter 1 - Literature Review

Chapter 2 - Published Original Manuscript

List of Tables

Chapter 1 - Literature Review

Chapter 2 - Published Original Manuscript

List of Abbreviations

ACA	Anti-centromere antibody
ACR	American College of Rheumatology
ADAM17	A disintegrin and metalloproteinase 17
AECA	Anti-endothelial cell antibody
ANA	Anti-nuclear antibody
ATA	Anti-topoisomerase I antibodies
AT_1R	Angiotensin type I receptor
BAFF	B cell activating factor
CREST	Calcinosis, Raynaud's, esophageal dysfunction, sclerodactyly, telangiectasia
CSRG	Canadian Scleroderma Research Group
CTD	Connective tissue disease
CTGF	Connective tissue growth factor
DAMP	Damage-associated molecular pattern
dcSSc	Diffuse cutaneous systemic sclerosis
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EC	Endothelial cell
ECM	Extra-cellular matrix
EGF	Epidermal growth factor
EGFR	EGF receptor

EMT	Epithelial-mesenchymal transition
Endo-MT	Endothelial-mesenchymal transition
EPC	Endothelial progenitor cell
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
ET_AR	Endothelin type A receptor
ET_BR	Endothelin type B receptor
EULAR	European League Against Rheumatism
Fra	Fos-related antigen
Fli1	Friend leukemia integration 1
GAVE	Gastric antral vascular ectasia
GERD	Gastroesophageal reflux disease
GVHD	Graft-versus-host disease
HB-EGF	Heparin-binding EGF-like growth factor
IFN	Interferon
IgG	Immunoglobulin type G
IL	Interleukin
ILD	Interstitial lung disease
IRF	Interferon regulatory factor
LAP	Latency-associated peptide
Klf5	Krüppel-like factor 5
lcSSc	Limited cutaneous systemic sclerosis
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MSC	Mesenchymal stem cell
MVEC	Microvascular endothelial cell
NF-κB	Nuclear factor-κB
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PF	Pulmonary fibrosis
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PKC	Protein kinase C
PM	Polymyositis
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
RA	Rheumatoid arthritis
RLR	Retinoic acid inducible gene-I-like receptor
RNAP	RNA-polymerase
ROS	Reactive oxygen species
RP	Raynaud's phenomenon

SLE	Systemic lupus erythematosus
SRC	Scleroderma renal crisis
SS	Sjögren's syndrome
SSc	Systemic sclerosis
Th	Helper T (cell)
TIMP	Tissue inhibitors of metalloproteinases
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor
Treg	Regulatory T (cell)
TSK1	Tight-skin (mice)
TSP-1	Thrombspondin-1
UCD	University of California Davis
U1snRNP	U1-small nuclear ribonucleic particles
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

Dedication

For Remy, my mother.

Mama, you are in all of these pages.

Acknowledgments

This MSc thesis is the result of a somewhat convoluted journey. It is not what I had planned, as I began this journey with the goal of completing a PhD. Many factors contributed to my decision to switch to a Master of Science degree, which I like to summarize as life having gotten "in the way". Thus, this journey involves much more than a Master's thesis, and numerous people have contributed to my experience in a positive way. I am deeply grateful to all those who have accompanied me on this adventure, even if I forget to mention you here.

First, I thank Dr. Marc Servant for having invited me to join his lab and embark on this project, for his mentorship, and for his patience, and for having introduced me to the wonderful world of cell signaling. And, I would not have met Marc, had it not been for Dr. Murray Baron. I am grateful to Dr. Baron for the opportunity to work in Systemic Sclerosis research and for setting me on the path to further graduate studies in this field. Also, I am grateful to the Canadian Scleroderma Research Group (or CSRG), under Dr. Baron's direction, for their student training program. This journey would have been impossible without the funding from the CSRG.

Thank you also to Dr. Baron's team, especially Maura Buchignani and Suzanne Taillefer. Importantly, I must recognize the patients and control subjects who participate in the CSRG. I know that much time and effort go into completing the stacks of questionnaires, not to mention giving your biological samples. I do not take any of this for granted!

I am grateful also for the support from my family - my parents, brother, sister-in-law, aunts, uncles, cousins. I have in my mind a cloud of miscellaneous words of encouragement that you have informally spoken to me over the years. They've stayed with me, and they helped me to keep going. Above all, I am grateful for my mother's interest, pride, and insistence. I know you would have been proud to see that I produced something at the end of all this, though of course I wish I could have done more. Thank you to Léa, Wesley and Marco for the day-to-day support.

And last but not least, at the Faculté de Pharmacie: Thank you to my lab colleagues and friends. I hold dear many memories of thought-provoking conversations, laughter, and general good times :) and I cherish the friendships that "happened" while we were doing science. Thank you to Dr. Denis deBlois and Dr. Daniel Levesque for taking the time to read, evaluate and provide feedback on my thesis. *Un gros merci* to Andrée Mathieu for answering my many questions and always being helpful and encouraging. And I appreciated so, so much Dr. Céline Fiset for your time, your kindness, and for wanting to help me move forward.

Chapter 1. Literature Review

1.1. Systemic Sclerosis (SSc)

Systemic sclerosis (SSc), also called "scleroderma", is a devastating multi-system disease of the connective tissue, with features of autoimmunity, inflammation, fibrosis of the skin and internal organs, and vasculopathy[1]. It is a relatively rare chronic progressive disease with a general prevalence reported to range from 30 to 489 per million[2-7] and an incidence of 0.6 to 122 per million per year[7]. Women are affected approximately 5 times more frequently than men[2,5], but men who have SSc tend to have more severe disease symptoms and increased mortality[8]. The etiology of SSc is complex and not well understood. There is no cure for this disease, although disease survival and morbidity have improved over the past decades due to improved diagnosis and early detection and management of serious complications like scleroderma renal crisis (SRC), gastro-esophageal reflux disease, digital ulcers and pulmonary arterial hypertension[9,10]. SRC was once the main cause of mortality, but since the advent of angiotensin-converting enzyme inhibitors, this is now a controllable complication[11]. Today, the main causes of death in SSc are pulmonary arterial hypertension and interstitial lung disease[12].

1.1.1. Clinical Manifestations

SSc is clinically a very heterogeneous condition. Disease manifestations and severity, as well as disease course progression vary greatly from one patient to another. A diagnosis of SSc is typically based on the judgment of a rheumatologist and the presence of particular signs associated with SSc, like skin tightness and hardness, Raynaud's phenomenon, and the presence of disease-specific anti-nuclear antibodies (ANAs). Since SSc is rare and complex, it sometimes takes a long time before patients receive a correct diagnosis.

Raynaud's phenomenon (RP) refers to transient ischemia in the extremities, for example, in the fingertips, due to vasospasm of the small blood vessels in response to cold or stress. RP is usually the first sign of a problem in SSc, and unlike other symptoms, virtually all patients (>95%) experience it[13]. Primary or idiopathic Raynaud's is not uncommon, however, so screening for other markers such as ANAs and abnormal nailfold capillaries is required if SSc is suspected. Unlike primary RP, which is thought to be due to a functional problem, namely an exaggerated central and/or peripheral nervous system mediated vasospasm[14,15], SSc-related Raynaud's results at least in part from structural abnormalities in the microvasculature and digital arteries[14,15]. SSc-related RP is severe, compared to primary RP which is reversible and does not lead to other complications.

Scleroderma, literally "hard skin", due to dermal inflammation and fibrosis, is another prominent symptom of SSc. In most cases of SSc, skin fibrosis first develops on the fingers and hands. Eventually, hard skin also appears on the lower extremities, forearms, face and neck. In the diffuse cutaneous subset of patients, the skin of the trunk, arms and legs may also become fibrotic. In addition to the tightening and thickening of the skin, patients experience intense pruritis, areas of hyper- and hypo-pigmentation, reduced hair follicles and reduced sweating in the affected skin.

Many SSc patients develop distressing changes to their appearance. Scleroderma of the face gives the skin an abnormal shiny and smooth appearance. Conspicuous pigmentary changes may occur, and distinct telangiectasias - red spots due to dilation of subcutaneous blood vessels - are also common on the face. The lips become thin and furrowing appears around the mouth. The mouth opening is severely constricted, which can hinder effective oral hygiene and professional dental care. The nose can become narrower and pinched[16,17].

The hands also undergo important changes. In addition to Raynaud's, stiffness and puffiness of the hands and fingers are another early sign of SSc. Later, almost half of SSc patients develop digital ulcers, a painful complication that seems to appear spontaneously, heals exceedingly slowly, sometimes over the course of weeks or months, and can lead to impaired hand function, osteomyelitis, autoamputation and gangrene[18]. Calcinosis (calcium deposits) in the soft tissues of the hands, as well as on the elbows and knees, is also common, and may lead to skin ulceration and infection[19]. Patients also experience joint pain and stiffness in their hands. Sclerodactyly (hard skin of the digits) is characterized by shiny, tight skin of the fingers, resulting in fingers or toes that are difficult to move or straighten. This may lead to flexion contractures, which refers to the digits becoming fixed in a flexed position. These complications in the hands result in pain and loss of function, and can be debilitating.

Internally, multiple organs are often affected by fibrosis and vascular problems, including those of the gastro-intestinal tract, lungs, heart, and kidneys. Patients experience a range of symptoms related to the extent and severity of internal organ involvement, such as: dry cough, dyspnea, chest pain, difficulty swallowing, bloating, weight loss, cachexia, malabsorption, hypertension, pain and fatigue, to name but a few[20]. In short, patient quality of life is greatly affected by serious complications that range from esophageal dysmotility and bacterial overgrowth, to potentially fatal problems like lung fibrosis, pulmonary arterial hypertension and renal failure with severe hypertension (SRC).

1.1.2. Classification / subtypes

The diverse clinical manifestations of SSc, and the extent of organ involvement and pattern of disease progression vary widely among patients. However, homogeneous groups of

patients can be identified. Depending on the particular constellation of symptoms and signs that are present, patients can be classified into distinct disease subtypes. These are primarily defined by the extent of skin fibrosis, which is also associated with specific patterns of visceral involvement[21]. The two main subsets are limited cutaneous SSc and diffuse cutaneous SSc (summarized in Table 1). Patient autoantibody profiles are also used to distinguish between the two subtypes. There are certain important vascular complications and organ involvement that occur in both groups, which affect disease severity and mortality[5].

1.1.2.1. Diffuse cutaneous SSc (dcSSc)

The diffuse cutaneous SSc (dcSSc) subtype is characterized by severe skin fibrosis on the torso as well as the extremities (hands, arms, face, and legs), and the presence of antitopoisomerase antibodies (ATA)[22]. This form of SSc carries a worse prognosis, with more extensive and more severe complications, and tends to have a more rapid onset and progression[4]. Diffuse SSc patients are more likely to have serious kidney and heart involvement, and such complications most often occur early in the course of the disease[23]. Scleroderma renal crisis is more likely to occur in this subgroup.

1.1.2.2. Limited cutaneous SSc (lcSSc)

Limited cutaneous SSc (lcSSc) is defined by skin fibrosis being distal from the elbows and knees[22], but often limited to the fingers (sclerodactyly). The skin of the face may also be fibrotic. This SSc subtype is sometimes referred to as CREST syndrome, named for the symptoms that these patients present: *c*alcinosis, *R*aynaud's phenomenon, *e*sophageal dysmotility, *s*clerodactyly and *t*elangieactases[21]. The presence of anticentromere antibodies

(ACAs) is also a marker for this subset[21,22]. Patients with lcSSc are more at risk of developing fingertip ischemia and ulcers, and pulmonary arterial hypertension (PAH), but despite significant morbidity their overall prognosis is better than patients with the diffuse form[21].

1.1.2.3. Other subtypes

Approximately 10% of SSc patients also express features of another connective tissue disease (CTD)[24], such as systemic lupus erythematosus (SLE), polymyositis (PM), rheumatoid arthritis (RA) or Sjögren's syndrome (SS). Patients with so-called SSc-CTD overlap syndome have been proposed to constitute a distinct subset[24,25], because their SSc resembles a version intermediate to lcSSc and dcSSc, and they possess autoantibody markers distinct from those ascribed to the lcSSc and dcSSc subgroups. These overlap phenotypes are associated with a unique gene signature[26] and with anti-U1-small nuclear ribonucleic particles (U1snRNP) antibodies[27,28].

1.1.2.4. Classification Criteria

Given the heterogeneous nature of SSc, some experts suspect that "systemic sclerosis" comprises a group of distinct diseases with shared features[29]. Although SSc may be considered to have two main subtypes - lcSSc and dcSSc - further subtyping of patients may be important for managing patients and their treatments, and for research purposes which often require objectively relatively "homogeneous" groups of patients. In order to do meaningful SSc research with sufficient statistical power, it is often necessary to involve patients from an expanded population. Patient data and biological samples need to be collected at different geographical centers by different researchers. (For example, the Canadian Scleroderma

Research Group (CSRG) was created for this reason, allowing rheumatologists from across Canada to establish a bank of Canadian patient biosamples and data, which may be shared among its members for research purposes.) To reduce the possibility of variability in definitions and judgments used by the diagnosing specialists, classification systems have been developed in order to establish criteria that will allow identification of homogeneous groups of SSc patients and comparisons among these groups. These criteria may inform diagnosis, although that is not their intended purpose[30,31] (Table 2). An earlier version had been used for many years, since 1980[30]. A new criteria definition was created and accepted in 2013 jointly by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) [31]. Studies in molecular subset definitions may be useful in clarifying the distinction between groups.

Table 1. Summary of limited cutaneous and diffuse cutaneous SSc phenotypes[21,32-36]

Features	**lcSSc**	**dcSSc**
Skin involvement	Limited to face and fingers, distal to elbows; gradual onset and slow progression; usually symmetric	Affects face, fingers, extremities, trunk; rapid onset and rapid progression; usually symmetric
Raynaud's Phenomenon	Precedes skin involvement; associated with critical ischemia	Onset coincident with skin involvement, may be mild
Musculoskeletal	Early arthralgia, fatigue	Severe arthralgia, carpal tunnel syndrome, tendon friction rubs
ILD	Sometimes present	Early and significant incidence
GERD	Present in 85%	Common
Pulm. Fibrosis	Occasional, moderate	Frequent, early and severe
PAH	Frequent, late, may be isolated	May occur, often in association with pulmonary fibrosis
SRC	Very rare	Occurs in 15%; early
Calcinosis cutis	Frequent, prominent	May occur, mild
SSc-specific ANAs	Anticentromere, anti-Th/To, Anti-PM-Scl, Anti-Ku, Anti-U1 RNP	Antitopoisomerase I, anti-RNA polymerase III, Anti-RuvBL1/2

Table 2. The ACR-EULAR Criteria for the classification of systemic sclerosis[31]

1. These criteria are applicable to any patient considered for inclusion in a SSc study.

2. These criteria are not applicable to:

a) Patients having a SSc-like disorder better explaining their manifestations, such as: nephrogenic sclerosing fibrosis, generalized morphea, eosinophilic fasciitis, scleredema diabeticorum, scleromyxedema, erythromyalgia, porphyria, lichen sclerosis, graft versus host disease, and diabetic cheiropathy. b) Patients with `*Skin thickening sparing the fingers*',

Items	Sub-items	Weight / Score
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints *(sufficient criterion)*		9
Skin thickening of the fingers^ *(only count the highest score)*	Puffy fingers Sclerodactyly of the fingers (distal to MCP but proximal to the PIPs)	2 4
Finger tip lesions^ *(only count the highest score)*	Digital Tip Ulcers Finger Tip Pitting Scars	2 3
Telangiectasia		2
Abnormal nailfold capillaries		2
Pulmonary arterial hypertension and/or Interstitial lung Disease* (*Maximum score is 2)	PAH ILD	2
Raynaud's phenomenon		3
Scleroderma related antibodies** (any of anti-centromere, anti-topoisomerasel	Anti-centromere Anti-topoisomerasel	3
[anti-Sd 70], anti-RNA polymerase III) (**Maximum score is 3)	Anti-RNA polymerase III	
	TOTAL SCORE^:	

Patients having a total score of 9 or more are being classified as having definite systemic sclerosis.

PAH is pulmonary arterial hypertension. The definition is proven PAH by right heart catheterization. ILD is interstitial lung disease defined as pulmonary fibrosis on HRCT or chest radiograph, most pronounced in the basilar portions of the lungs, or presence of `velcro' crackles on auscultation not due to another cause such as congestive heart failure. See definition of terms for all variables (Table 2).

^Add the maximum weight (score) in each category to calculate the total score.

1.1.3. SSc Etiology

Although the etiology of SSc is not known, it is believed to involve some environmental trigger in genetically susceptible individuals. In the general population, the SSc incidence rate is 0.026%, while in families with a history of SSc, the incidence is 1.5-1.7%[37]. Different genetic variants can be linked to specific clinical patterns. For example, African American SSc patients are more likely to have pulmonary fibrosis and anti-topoisomerase I, anti-fibrillarin, and anti-RNP autoantibodies, compared with other groups[38], and in the case of the Choctaw Native American population, which has a higher SSc prevalence than any other group[39], SSc tends to be relatively homogeneous for the dcSSc subtype with pulmonary fibrosis and ATAs[39]. However, concordance among monozygotic twins is low. Genome-wide association studies have identified an HLA II region that associates strongly with SSc[40], and single nucleotide polymorphisms that confer susceptibility to SSc have been identified in the genes coding for interferon regulatory factor 5 (IRF5)[41-43], IL-1α[44], TGF-β[45], fibrillin-1[46], tumour necrosis factor-α (TNF-α)[47], connective tissue growth factor (CTGF)[48], and others.

A number of environmental triggers are suspected as potential causative agents of SSc. Exposure to certain chemicals has been associated with SSc development, including silica dusts, benzene, trichloroethylene, vinyl chloride, epoxy resins, and others[49]. The cancer drug bleomycin, which is also used to induce skin and pulmonary fibrosis in mice, also has been implicated[49]. Exposure to and/or infection by specific microbial pathogens has been linked with SSc[50], based on high titres of antibodies against parvovirus B19[51], human cytomegalovirus[52] and *Helicobacter pylori*[53] in SSc patients. A connection with cancer has also been reported, in that antitumour immunity may bring about SSc in patients with anti-RNA-Polymerase III autoantibodies[26].

Another hypothesis, which may explain the origin of autoimmunity in SSc, lies in the concept of microchimerism[54], which refers to the presence of cells in one individual that originate from another genetically different individual. This may occur naturally by the persistence in maternal tissues of fetal cells that have passed the placenta during pregnancy[54-56], conversely by maternal cells that persist in the offspring long after birth[54,57], or in the case of a transplant or transfusion. Evidence of a greater degree of michrochimerism has been found in female SSc patients than in controls[58,59]. The persistence of fetal cells and DNA in the mother may relate to the increased incidence of SSc in women in the years after childbearing[60] (peak age at onset is 20-50 years[61]).

1.2. SSc Pathophysiology

The clinical manifestations of SSc result from underlying pathologies that can be categorized according to four interrelated "hallmarks" of SSc, namely fibrosis, vasculopathy, inflammation, and autoimmunity. Aberrant connective tissue metabolism, culminating in fibrosis of skin or major organs, has traditionally been considered to be the primary problem in SSc. Currently, despite the lack of a single unifying theory that explains all of the typical SSc features, it is now hypothesized that SSc pathogenesis follows a typical sequence of events, possibly initiated by vascular dysfunction, which progresses to inflammation, immune cell activation and autoantibody production, all of which then contribute to profibrotic processes[62]. However, the interplay of these events is also important. For example, chronic inflammation and cytokine release cause further microvascular damage[63], and fibroblasts, the cells that drive fibrosis, can also have proinflammatory activity[64,65] (Fig. 1).

Interestingly, there is much more widespread inflammation and consequent fibrosis in dcSSc than in the limited cutaneous form. On the other hand, SSc vasculopathy is much more prominent in lcSSc, as evidenced by the earlier onset of Raynaud's and the vascular complications associated more frequently with this subtype, such as digital ulcers, PAH and SRC. This suggests that the pathogenesis of the two subtypes may be subtly distinct, although the sequence of events appears to be common.

What follows is a description of the pathophysiology underlying each hallmark feature and the corresponding cellular and molecular mechanisms involved. Although these four disease components are described individually, the separation into the four hallmarks is artificial, as these processes are intimately interrelated.

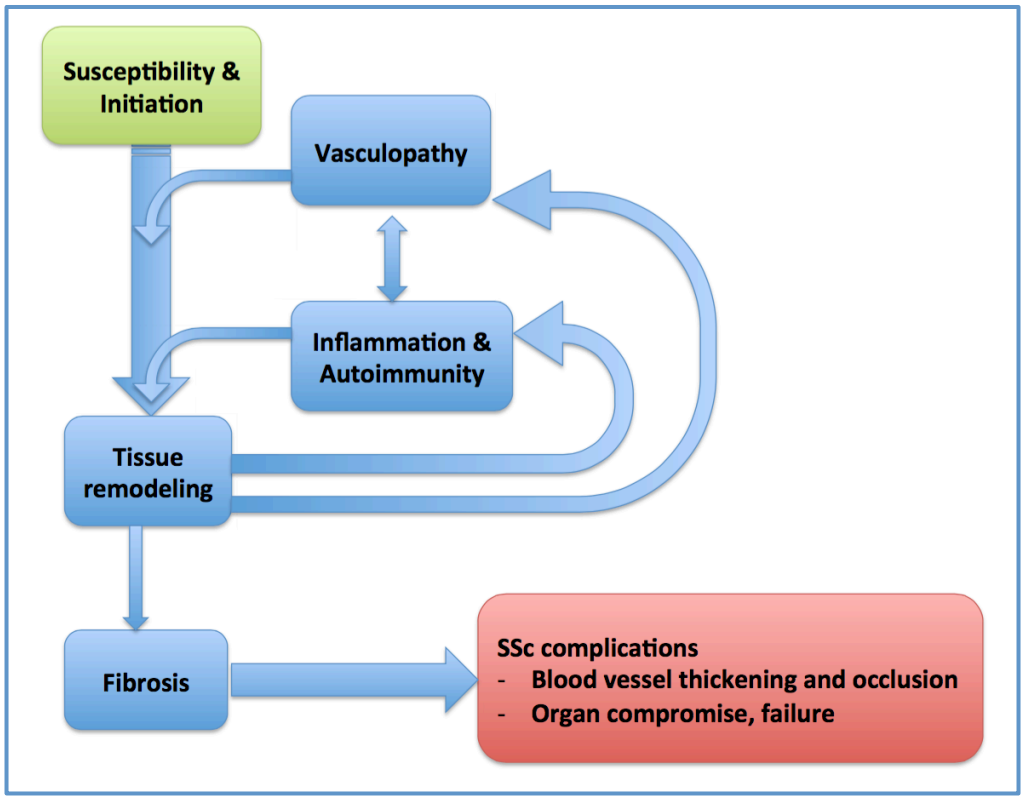

Figure 1. Overview of SSc pathogenesis (Adapted from Abraham & Distler (2007)[66]).

1.2.1. SSc Vasculopathy

The vascular manifestations of SSc, which constitute a major identifying feature, tend to occur early, prior to any signs of fibrosis. The vascular system, especially the microvasculature is compromised in several important ways[67]. Specifically, SSc patients have a marked decline in capillary density, insufficient angiogenesis, and intimal proliferation. Endothelial cell (EC) dysfunction appears to be a ubiquitous early event in SSc. It has been suggested that some form of microvascular injury may be the initiating factor in SSc pathogenesis[68].

1.2.1.1. Decreased Capillary Density

EC injury and apoptosis occur early and are speculated to be initiating events in SSc[69] pathogenesis. Patients have progressive changes in their capillary beds, ranging from abnormal capillaries to avascular areas. This decreased density of capillaries has been reported in the skin and other organs affected by SSc, including lungs, heart, kidneys, muscles[67,70-74]. Structural abnormalities and progressive reduction in capillary density increase with disease progression to the extent that more than 50% of expected capillaries have been reported as absent[75,76]. In the nailfold (the base of the finger nail) it is possible to observe non-invasively the capillaries near the surface of the skin. Distorted and micro-hemorrhaging capillaries as well as areas of low capillary density observed by nailfold video capillaroscopy (Fig. 2), are characteristic of SSc. Indeed, abnormal nailfold capillaries are a sign pointing to an SSc diagnosis in patients presenting with RP. It is possible that these abnormalities near the skin surface are also representative of the blood vessel pathology at the internal organs, which show reduced blood flow and tissue hypoxia[77].

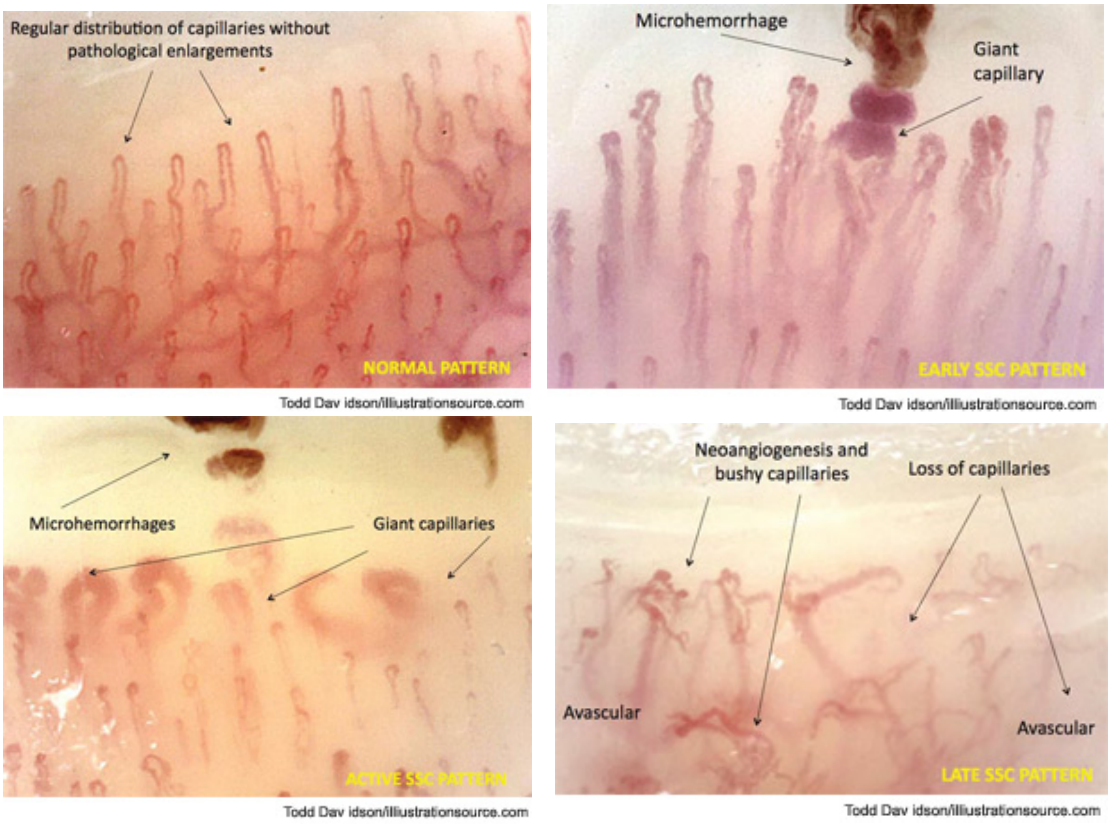

Figure 2. Nailfold video capillaroscopy images depicting normal capillaries compared to the capillary abnormalities typically observed in SSc. (magnification x200) (Images available online from http://www.the-rheumatologist.org/article/capillaroscopy-a-safe-and-direct-method-for-ssc-diagnosis/).

1.2.1.2. Endothelial Injury

Endothelial dysfunction plays an important role in SSc vasculopathy. The endothelium is a complex organ consisting of a single layer of endothelial cells which make up the capillaries and the inner lining of other blood vessels. It has important roles in regulating blood flow and homeostasis of the vascular wall. Its endocrine, paracrine and autocrine activities include regulating coagulation and fibrinolysis, vascular permeability, vascular tone, metabolism and

nutrition of surrounding cells[66]. The endothelium reacts to and produces a variety of mediators important for vasodilation (like nitric oxide (NO)), vasoconstriction (like endothelin), and other functions. ECs also are important for maintaining VSMCs in a quiescent state[78]. Endothelial dysfunction, refers to an abnormal “activation" in which there is a disturbance in the balance of vasoactive factors, resulting in increased vasoconstriction, and also a proinflammatory and pro-coagulant state.

A variety of agents have been proposed that potentially induce injury and/or activation of the microvascular endothelium early in the SSc disease process, such as pathogens[50-53], immune-mediated cytotoxity, ischemia/reperfusion and generation of reactive oxygen species (ROS)[79-81], and anti-endothelial-cell autoantibodies (AECAs)[82-84]. AECAs have been reported in a significant proportion of SSc patients[82,83,85], and can upregulate EC adhesion molecules and induce apoptosis[82,83,86,87]. Similarly, ROS may cause vascular endothelial damage, and SSc patients were noted to have a significant reduction in plasma antioxidant capacity[80].

Subsequent to microvascular injury, the ensuing events include endothelial and smooth muscle cell proliferation, damage to the internal elastic lamina, inflammatory infiltration of the vessel wall and fibrosis, leading to obliterative vasculopathy and significant tissue hypoxia[88]. Oxidative stress and ischemia further exacerbate vessel wall damage. Once endothelial dysfunction ensues, the delicate balance of EC-derived vasoactive mediators is disturbed, promoting an anti-angiogenic, pro-inflammatory and profibrotic state in SSc. For example, thrombospondin-1 (TSP-1), an antiangiogenic cytokine that induces EC apoptosis and inhibits their proliferation, is released in high concentrations by hypoxic ECs, and has accordingly been reported to be present at elevated levels in SSc samples compared to controls[89].

1.2.1.3. Endothelin-1

Endothelin-1 (ET-1) is an important mediator of vasoconstriction, which acts on the type A and B endothelin receptors (ET_AR and ET_BR), and is primarly produced by ECs. SSc patients have an imbalanced expression of endothelin receptors[66,90]. In addition, ET-1 is overexpressed in SSc skin, lungs, liver and kidney tissues and in the circulation[91-96]. ET-1 also has been shown to induce a profibrotic phenotype in fibroblasts, suggesting a possible role of ET-1 in both SSc vasculopathy and fibrosis[90]. The use of the dual endothelin receptor antagonist, bosentan, has confirmed the importance of this target in SSc pathology. Bosentan was shown to improve the impaired vasodilation of tight-skin (TSK1) mice[97], an experimental mouse model of SSc.

ET-1 also acts on VSMCs, inducing contraction and proliferation, which may contribute to SSc PAH and digital ulcers. In clinical studies, bosentan appeared to have some ability to prevent digital ulcers and to treat SSc-related PAH[98-100], and to slow the progression of changes to the nailfold microvasculature over a 3-year period[101].

1.2.1.4. Insufficient Angiogenesis

Related to the problem of decreased capillary density is an insufficient angiogenic and vasculogenic response[102-105]. Normally, the hypoxic conditions resulting from capillary damage and reduced capillary density spur an increased production of proangiogenic mediators, which would stimulate the repair and regeneration (angiogenesis) or *de novo* generation (vasculogenesis) of blood vessels. In SSc, vascular regeneration processes are defective[102-104]. Studies in SSc peripheral blood mononuclear cells (PBMCs), SSc PBMC supernatants and SSc sera have demonstrated severe disturbance in angiogenic and

vasculogenic factors[103,106-108]. However, the lack of new blood vessel formation is not due to a diminished production of proangiogenic mediators, as many proangiogenic factors are upregulated in SSc, including sVCAM-1, sE-selectin, E-selectin, endothelin-1, VEGF and VEGF receptors[103,105,109,110].

1.2.1.5. Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF, also called VEGF-A) is the prototype member of the angiogenic VEGF cytokine family. VEGF signals through two related receptor tyrosine kinases, VEGFR-1 and VEGFR-2. This growth factor has important implications in both physiological and pathological angiogenic processes[111].

In SSc, VEGF has been detected in blood and skin at greater levels than in a healthy population at different stages of disease progression[109,110,112], and both VEGFR-1 and VEGFR-2 are overexpressed in SSc[110,113]. Interestingly, the increased levels of VEGF correlate with the severity of blood vessel loss and damage, and SSc disease progression, and not with increased blood vessel repair and regeneration. This may be explained by the fact that translation of *VEGF-A* mRNA can result in different splice variants, some of which are pro- and others anti-angiogenic[114,115]. Indeed, Manetti *et al.* have shown a greater amount of antiangiogenic isoform $VEGF_{165}b$ in SSc circulation and skin than in healthy or other disease controls[116] and that the increased amount of $VEGF_{165}b$ correlates with the degree of capillary abnormality and disappearance[117]. They have also reported that microvascular endothelial cells (MVECs) isolated from SSc patients constitutively express higher levels of antiangiogenic $VEGF_{165}b$ in culture than do control MVECs[116]. The two $VEGF_{165}$ splice variants have equal affinity for VEGFR-2, but the antiangiogenic "b" isoform fails to induce

complete receptor phosphorylation, and results in attenuated downstream signaling. Thus the $VEGF_{165}b$ isoform acts as an endogenous inhibitor. Interestingly, the alternate splicing of the antiangiogenic VEGF isoform may be driven partly by TGF-β[116] (a cytokine considered to be the master regulator of fibrosis, discussed in Section 1.2.4.2).

1.2.1.6. Endothelial Progenitor Cells & Mesenchymal Stem Cells

In addition to deficient angiogenic pathways, the amounts of circulating vasculogenic cells are also abnormal. In SSc, bone-marrow derived endothelial progenitor cells (EPCs), which participate in vascular repair, have been found to be decreased in some studies[105], and increased in others[118-120]. The significance of the contradicting reports is not clear, but diminished numbers of EPCs would result in reduced vasculogenesis, while an expanded presence may be explained as EPCs homing to increased levels of angiogenic factors, but then having reduced capacity to regenerate. Indeed, SSc EPCs, including mesenchymal stem cells, have impaired migratory responses to VEGF, in addition to early senescence[121] The giant and other abnormal capillaries observed in patient nailfolds (Fig. 2), as well as other commonly observed SSc features like telangiectasia of the skin and of the gastrointestinal mucosa (including gastric antral vascular ectasia (GAVE))[122], are likely the result of failed efforts to build new vessels in response to the proangiogenic stimuli[103].

1.2.1.7. Vascular Wall Remodeling in SSc

Vascular smooth muscle cells (VSMCs) constitute the majority of cells in the medial wall. VSMCs of the media are normally present in a differentiated contractile state, and regulate vascular tone and thus blood flow. The endothelium has a role in maintaining VSMCs

in this quiescent state[123]. Infiltrating leukocytes and ECM components also influence VSMC activation[124,125]. Following endothelial dysfunction, VSMCs dedifferentiate into a synthetic phenotype. In this state, they proliferate and secrete collagens and other ECM proteins[126,127]. Intimal proliferation and fibrosis of blood vessel walls, a striking feature of SSc vasculopathy, further exacerbate the diminished blood flow due to absent and damaged microvessels[128,129]. In larger blood vessels, there is evidence of luminal narrowing, and sometimes complete obliteration, due to VSMC proliferation in the intima and the deposition of collagen and other ECM components[76]. In addition, normal intimal functions are disrupted, as evidenced by increased coagulation and decreased fibrinolysis[130], again impeding blood circulation. These features clinically manifest as the vasculopathic complications typical of SSc: RP, telangiectasias, DUs, GAVE, PAH, myocardial dysfunction, SRC.

1.2.2. Immune Dysregulation

Dysregulation of the immune system is another hallmark feature of SSc. The immune system has classically been divided into two parts: the innate immune system which provides immediate non-specific defenses against disease; and the adaptive immune system, which acquires memory of encountered infections and can launch enhanced defenses to target specific pathogens or foreign entities. The innate immune system also has important functions in regulating and activating players of the adaptive immune system, and likewise, adaptive immune cells and their products can recruit and influence activity of innate immune cells. This makes for an intricate but powerful system of defense, and a complicated pathology in the case of SSc.

Chronic inflammation and autoimmunity are both significant and important components of SSc. In early stages of SSc there is evidence of inflammatory cell infiltrates, degranulation of mast cells and peripheral blood mononuclear cells with an activated phenotype[131,132]. As inflammation in skin precedes fibrosis, and given the normal role of inflammation in immune responses and wound healing, inflammatory processes likely are important in driving the pathogenesis of SSc, although the underlying mechanisms are not entirely clear. In addition, pathologic activation of the adaptive immune system is indicated by both B and T-cell abnormalities.

1.2.2.1. Inflammation

Inflammation is a complex protective mechanism that is an important component of the innate immune system's response to injury or infection. An injury or sign of a pathogen will often trigger an inflammatory response, which is initiated by cellular effectors of the innate immune system when they recognize pathogen-associated molecular patterns (PAMPs) and/or danger-associated molecular pathogens (DAMPs) through their pattern recognition receptors (PRRs). There are different classes of PRRs, and they include membrane-bound, cytosolic and secreted proteins. Toll-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene I (RIG-I)-like receptors (TLRs, NLRs, and RLRs, respectively) are all examples of PRR families. Specific PAMPs (such as lipopolysaccharide (LPS), or double-stranded RNA (dsRNA), for example, which betray the presence of gram-negative bacteria, or replicating virus, respectively) or DAMPs (*e.g.* cellular contents not normally present in the circulation, signaling tissue injury) will be detected by their cognate PRRs (TLR4, TLR3, TLR2 and TLR4, respectively). Activation of these receptors typically

causes a coordinated cascade of cellular events leading to the transcription of genes involved in inflammation and anti-microbial defense. In the case of TLRs, adaptor molecules such as MyD88 or TRIF are recruited[133], which then initiate interferon regulatory factor (IRF) or nuclear factor κ-B (NFκB) signaling pathways, leading to the induction of inflammatory and anti-microbial mediators (cytokines, interferon (IFN)), as well as chemokines that recruit and activate circulating leukocytes to the site of inflammation[133,134]. These cells subsequently produce more cytokines and other mediators, some of which serve to dilate and increase the permeability of blood vessels, allowing larger circulating white blood cells and the other circulating mediators to access the inflammatory site. Further, other mediators, like chemokines (cytokines that guide cell migration) recruit the necessary cells, such as fibroblasts or antibody producing plasma cells. PRR signaling, and ensuing cytokine production, also has a role in recruitment and antigenic priming of naïve T-cells. Yet other cytokines are important for stimulating the adaptive immunity arm of the immune system to produce B cells and T Cells. The influx of blood which brings circulating immune cells and mediators to the inflammatory site, leads to redness and swelling, pain due to the pressure of the swelling as well as stimulation of pain receptors. These, along with warmth at the inflammation site, attributable to increased local circulation, constitute Celsius' four classic manifestations of inflammation: *rubor*, *tumor*, *dolor*, *calor*[135].

In recent years, the role of TLRs in SSc has gained appreciation. TLR signaling is known to lead to Type I IFN (IFNα/β) production as well as that of many other cytokines. TLR activation has also been related to fibroblast activation[136]. Patients with SSc, similar to those with other autoimmune connective tissue diseases, have a Type I IFN gene expression

pattern or signature[137,138]. However, further studies are needed and ongoing in order to elucidate the exact role of TLRs and other PRRs in SSc.

1.2.2.2. Chronic Inflammation

Acute inflammation is characterized as having an immediate onset in response to a pathogen or trauma and a short duration, in the order of days. Chronic inflammation, on the other hand, occurs over a long period of time. Chronic inflammation is also characterized by the migration of large amounts of mononuclear cells, like lymphocytes, while in acute inflammation, polymorphonuclear cells, like neutrophils, infiltrate in large numbers. In chronic inflammation there is ongoing destruction of tissue, concurrently with persistent attempts at tissue repair. Once the pathogen is cleared or the tissue damage is controlled, anti-inflammatory cytokines and other endogenous regulatory signals guide the termination of inflammation. Evidence of the resolved inflammatory response may remain, in the form of circulating cells of the adaptive immune system guarding against a recurring infection and/or scar tissue formation. Problems at different phases of the inflammatory response - such as the failure to eliminate a pathogen or heal damaged tissue (and thereby continuous exposure to PAMPs and DAMPs), excessive pro-inflammatory cytokines and other mediators, dysfunctional or insufficient anti-inflammatory mediators, fibrosis or other tissue damage that signals further danger and triggers more inflammation, just to name a few - perpetuate damage to tissue and cells and production of B and T cells. The chronic inflammation in SSc is thus intimately linked with the other hallmarks of autoimmunity and fibrosis.

1.2.2.3. Immune Dysfunction in SSc

The presence of inflammatory infiltrates[131,132] is an early characteristic in SSc, in addition to the early vascular events discussed above (Section 1.2.1) As described earlier, endothelial dysfunction is likely at the root of the early vasculopathy and precedes the inflammation[120,139,140]. As one of many feed-forward cycles in SSc pathophysiology, beyond its vasoconstrictive effects, ET-1 also behaves as a pro-inflammatory cytokine[141]. The inflammation then promotes further vascular injury. The edematous-inflammatory phase associated with SSc tends to diminish as the disease progresses to fibrosis.

Many studies have identified marked lymphocytic infiltration in symptomatic SSc tissues[142,143]. Histological analyses of skin biopsies from early dcSSc reveal accumulation in subcutaneous tissue of mononuclear cells, especially macrophages, mast cells and T cells[140,144-146], and predominantly activated CD4+ T cells[139,147,148]. Many abnormalities have been reported for specific subsets of CD4+, or T helper (Th) cells, in SSc. The balance of Th1/Th2 cells is skewed towards Th2 in SSc, and accordingly, SSc patients have increased levels of Th2-derived cytokines in their serum[149]. Th2 cells produce abundant pro-fibrotic and anti-inflammatory, including interleukin-4 (IL-4), IL-5, and IL-13[63], and also drive some of the vascular changes[140]. Pro-inflammatory Th-17 cells, another subset of CD4+ T cells, secrete great amounts of IL-17 and IL-22, and appear to be pathogenic in inflammatory autoimmune disease processes[150]. Th-17 cell counts are elevated in SSc compared to normal blood samples[150,151]. IL-1β, secreted by activated macrophages, drives Th17 differentiation[152]. Another CD4+ T cell subset, regulatory T cells (Tregs) suppress effector T cell proliferation, and as such have an important role in maintaining self-tolerance[153]. Amounts and function of Tregs are also abnormal in SSc. Both higher and lower amounts of Tregs have been reported

in SSc, but in general, these appear to have reduced suppressive function[154-156] and impaired Treg activity is associated with SSc disease severity[154,155,157]. Several cytokines, IL-1β, IL-2, IL-23 and TGF-β can induce Treg transdifferentiation into Th17 cells[156,158]. A shifting of Treg towards Th17 is one mechanism by which Treg numbers may be reduced, and at the same time Th17 cytokines are increased, thus contributing to increased immune activation and onset of autoimmunity[159]. Interestingly, an unusual "double positive" CD4+CD8+ T cell population that secretes excessive IL-4 has also been reported in SSc[160].

The degree of lymphocyte accumulation has been shown to correlate to SSc skin fibrosis severity and progression[161]. T cells are present at an early stage at sites undergoing fibrosis[140,145,162]. In graft-versus-host disease (GVHD), which mirrors many aspects of SSc and is thus used as a model to study this disease, rats develop skin lesions characterized by mononuclear cell infiltrates and subsequent skin fibrosis. Also some of the most common therapies used in SSc are immunosuppressives like cyclophosphamide, which target T and B cells. Despite having no known direct effects on fibroblasts or ECM production, cyclophosphamide has been shown to reduce progression of SSc-ILD (interstitial lung disease) and to reverse severe diffuse skin fibrosis[163,164].

Monocytes (which differentiate into myeloid dendritic cells and macrophages) and macrophages are also present in SSc inflammatory infiltrates in involved lung or skin[165,166]. Different classes of macrophages have been identified, and generally, macrophages may be classed as M1 type, which are important producers of pro-inflammatory cytokines, or M2 type, which are considered to have anti-inflammatory and fibrogenic properties. In SSc, evidence suggests an increased presence of pro-fibrotic, anti-inflammatory M2-type

macrophages. In addition, SSc monocytes have pro-fibrotic features, such as reduced levels of caveolin-1 which is a negative regulator of the pro-fibrotic cytokine TGF-β.

Dendritic cells, which express many PRRs and function as professional antigen-presenting cells are abundant producers of cytokines, and are important for the differentiation of naïve T cells into Th2 effectors, which then in turn are important cytokine producers. Dendritic cells also have a role in supporting self-tolerance of T-cells, thus defects in this role may be important in the development of autoimmunity. Early SSc dendritic cells have been shown to respond to TLR stimulation by producing greater amounts of IL-6 and TNF-α, two important pro-inflammatory cytokines, and less IL-10 and IL-12[167], as compared to late SSc or control cells. SSc dendritic cells have also been shown to have a pro-inflammatory gene signature.

1.2.2.4. Cytokines

Cytokines are a diverse group of signaling molecules that are produced by immune system cells, most significantly by macrophages and helper T (Th) cells, but many other cell populations as well. They transmit intercellular messages in autocrine, paracrine, or endocrine fashion. They mediate innate and adaptive immune responses and induce specific cellular responses upon binding their cognate receptors in their target cells. Cytokines can be categorized based on shared elements of their cognate receptors: some of the major cytokine groups are the chemokines, IL-1 family, IL-17 cytokines, Type I cytokines, Type II cytokines (interferons), TNF receptor family, TGF-β family [168]. Growth factors may be considered a sub-family of cytokines. Cytokines can also be classed by their prototypic and most characterized function (Table 3), although they tend to have pleiotropic and redundant

activity[169], making these functional categorizations artificial, albeit useful. For example, the anti-inflammatory cytokine IL-13 has a "main" function of reducing inflammation, but it also has important profibrotic activity (reviewed by Raja & Denton, 2015[170]).

Evidently, many cytokines are implicated in SSc pathology. It is beyond the scope of this thesis to discuss them all in detail. Some of the best characterized ones are summarized in Table 3 or explained in further detail in other sections of this thesis.

One cytokine that is of great interest in SSc is Interleukin 6 (IL-6). This pleiotropic cytokine is elevated in SSc skin and serum, both in early and long-standing disease[171]. IL-6 is a driver of chronic inflammation and endothelial cell activation. It has been shown to prevent T cell apoptosis[172], one mechanism by which it contributes to chronic inflammation. It also stimulates immunoglobulin production by activated B cells[173], pointing to an additional role in autoimmunity. IL-6 can affect fibroblast proliferation and induce synthesis of ECM components, like collagen I[172]. In addition, fibroblasts from SSc skin lesions constitutively produce higher amounts of IL-6 than fibroblasts from uninvolved skin or from healthy controls, giving rise to an autocrine positive feedback system[172]. In a 2-patient SSc study, tocilizumab, an anti-IL-6 receptor antibody, reduced skin hardness and collagen, and improved kidney function (but not lung fibrosis)[174], affirming the importance of this cytokine in SSc pathogenesis.

IL-1α is primarily a proinflammatory cytokine, whose mRNA is constitutively expressed in SSc fibroblasts[175]. It has autocrine activity, and induces the production of other pro-inflammatory mediators in endothelial cells, fibroblasts and dendritic cells, such as IL-6, IL-8, IL-12, ET-1, TNF, growth factors, and adhesion molecules (reviewed by Kawaguchi[176]). It stimulates the proliferation and differentiation of T cells[176]. Interestingly, two *IL1A* gene

polymorphisms have been found to have associations with SSc in a Japanese population study[44,177].

Table 3. Examples of cytokines classed according to their primary functions[169,170,178]

Cytokine function	**Examples**
Pro-inflammatory	IL-1, IL-1β, IL-6, IL-12, IL-18, TNFα, IFN-γ, GM-CSF
Anti-inflammatory	IL-10, IL-13, TGF-β, IL-22, IL-1Ra, IFN-α
Pro-fibrotic	IL-1, IL-1β, IL-4, IL-6, IL-17, TGFβ, FGF, HGF, BMP, PDGF
Anti-fibrotic	IFN-γ, IL-10
Chemotactic	CC and CXC chemokines, IL-8, MCP-1, MIP-1α
Angiogenic	IL-1, IL-6, IL-8, VEGF

1.2.2.5. B Cells in SSc

B cells are also prominent in SSc involved tissue infiltrates, and are present in greater amounts in SSc skin as compared to normal skin[179]. Not surprisingly, B cells are important players in SSc pathogenesis. Multiple B cell genes have been associated with increased susceptibility to SSc in genome-wide association studies (GWAS)[180]. In addition to their antibody-producing function, B cells are directly involved in antigen presentation, cytokine production, activation of T cells and dendritic cells[181], thus dysfunctioning B cells contribute to inflammation and fibrosis[182] via secretion of pro-inflammatory and pro-fibrotic mediators.

B cells also can stimulate pro-fibrotic activity in SSc fibroblasts by a mechanism that requires direct cell-to-cell contact and TGF-β, and that is heightened by the presence of B-cell activating factor (BAFF)[183]. However, it is their role as generators of autoantibodies that has received the most attention. The high levels of IL-6 in SSc stimulate the polyclonal B cell expansion noted in SSc[184], and SSc B cells appear to be chronically activated based on increased levels of positive response regulating molecules, such as CD19[185-187].

1.2.3. Autoimmunity in SSc

SSc is considered an autoimmune disease because virtually all SSc patients (more than 95%)[36] harbor high titres of ANAs, and many clinical features of SSc are common with other autoimmune systemic conditions, such as SLE, RA, MCTD, *etc*. Such autoantibodies exist in healthy populations as well, but at much lower levels[188]. A subset of SSc autoantibodies are associated very closely with particular SSc phenotypes, allowing their use as diagnostic and prognostic biomarkers. Generally speaking, there is very little overlap in ANA autoantibody profiles that are associated with limited or with diffuse SSc, or that differentiate SSc from related autoimmune rheumatic disorders (Table 1). In addition, SSc patients are known to harbor other autoantibodies that target self-antigens. Some of these have pathogenic functions with molecular mechansims that have been somewhat defined, or at least hypothesized. However, the passive transfer of antibodies has never been shown to cause development of an SSc phenotype.

The major ANAs that are specific to SSc include anti-topisomerase I (ATA), anti-centromere (ACA), anti-Th/To, anti-RNA polymerase III (anti-RNAPIII) and anti-fibrillarin (Table 4). These can reliably be used to identify disease subtype and predict disease

progression[189]. ATAs (also called anti-Scl-70 antibodies) target topoisomerase I, are correlated with dcSSc (in 30-40% of dcSSc patients), and very rarely are detected in lcSSc or other systemic autoimmune rheumatic diseases (SARDs)[189]. Presence of ATAs points to a poorer prognosis and more severe complications. ACAs, or anti-CENP antibodies are associated with the lcSSc subset (in 80-90% of lcSSc patients, and in less than 10% of dcSSc) and although overall morbidity is better than in the ATA-associated subset, ACA-positive patients are more likely to develop and die from PAH[189]. ATA and ACA rarely coexist in the same patient.

The mechanisms underlying auto-antibody production in SSc are not well understood. One hypothesis to explain the presence of autoantibodies that target intracellular nuclear antigens not normally exposed to circulating B lymphocytes is that these antigens may become exposed through some form of cell damage or defective apoptosis. Some environmental factor, perhaps a viral infection, may bring about cell damage and/or apoptosis in a setting with defective apoptotic processes. Increased levels of autoantigens in the circulation due to prolonged apoptosis or improper clearance of apoptotic material could lead to nuclear antigens being exposed to the immune system and subsequent auto-immunization, as has been reported in SLE[190]. Alternatively, oxidative modifications of autoantigens within apoptotic blebs[191] may render them immunogenic, leading to the production of autoantibodies. Other proposed mechanisms for autoantigen-driven autoimmunity include the exposure of normally hidden epitopes after proteolytic cleavage[192], or increased antigen expression in affected tissues[193].

The roles or effects of autoantibodies targeting their autoantigens are also poorly elucidated. It is generally accepted that anti-endothelial cell autoantibodies (AECAs) have a role in SSc, inducing endothelial cell apoptosis in early disease. However, the target epitope and mechanism by which this occurs is not clear. Similarly, anti-fibroblast autoantibodies have

been identified, but again, the molecular events responsible for autoantibody-induced fibroblast activation remain unclear. Autoantibody-autoantigen immune complexes may be internalized by plasmacytoid dendritic cells, and subsequently stimulate endosomal TLRs and an interferon response[194]. More recently, autoantibodies that target cell surface receptors have been reported, which may be directly pathogenic and have agonistic or antagonistic effects leading to SSc manifestations (some of which are summarized in Table 5). For example, anti-PDGFR, anti-ET_AR, and anti-AT_1R autoantibodies have been shown to activate signaling cascades initiated by their cognate autoantigens. Given that these reports are more recent, many of these functional effects are regarded as somewhat controversial.

1.2.3.1. Anti-PDGFR autoantibodies

SSc patients have been reported to harbor anti-PDGFR autoantibodies that recognize native PDGFR-α[81] and induce its phosphorylation and activate down-stream signaling and profibrotic gene expression[81]. Strikingly, these anti-PDGFR autoantibodies were noted to be exclusive to SSc patients and present in all SSc patients of the cohort studied. Further investigations have since demonstrated stimulatory anti-PDGFR autoantibodies in patients with chronic GVHD[195] as well as in SLE[196], two conditions with similarities to SSc. However, others have found no significant difference between SSc patients and healthy controls, in terms of the presence of anti-PDGFR autoantibodies and that these autoantibodies do not have any PDGFR-activating properties[197,198]. Similarly, Classen *et al.* (2009)[199] have reported an absence of functional PDGFR-directed antibodies in SSc sera. Some technical experimental differences may partly explain the contradicting results. For example, Gabrielli *et al.*[200] have pointed out that the myeloid and endothelial cells used by Classen *et al.*[199] and Loizos *et al.*[197], respectively, may

interact with the putative PDGFR-targeting autoantibodies differently than fibroblasts as reported by Baroni *et al*.[81], due the presence of Fc receptors which may capture the stimulatory autoantibodies, thus inhibiting their stimulatory activity[200]. These non-concordant results serve to underline that this interesting theory requires further study.

Table 4. Antinuclear antibodies relatively specific for SSc (Adapted from Chung and Utz (2004)[36], Kuwana and Medsger (2017)[201], and Mehra *et al.* (2013)[189]

ANA	**Target [main function of target]**	**Clinical association and phenotype**
Anticentromere	CENP-A, CENP-B, CENP-C [Separation of chromosome]	**Limited SSc** (80-90% of patients); PAH, severe peripheral vasculopathy, SRC.[202,203]
Anti-Th/To	RNase P/RNase MRP [small RNA/tRNA processing]	**Limited SSc**; small bowel involvement, hypothyroidism, ILD, PAH, SRC [204-207]
Anti-Ku	Ku80 and Ku70 [DNA repair]	**Limited SSc**; myositis overlap
Anti-topoisomerase-I (Anti-Scl-70)	DNA topo-isomerase I [uncoiling of DNA]	**Diffuse SSc** (30-40% of patients); severe peripheral vasculopathy, ILD/PF, cardiac involvement. [203,208]
Anti-RNAP I	RNA polymerase I	**Diffuse SSc**; rapidly progressive disease, severe internal organ fibrosis, SRC. [209,210]
Anti-RNAP III	RNA polymerase III components [small nuclear RNA transcription]	**Diffuse SSc**; Rapid progression, SRC, malignancy
Anti-PM-Scl	Exosome complex containing PM-Scl-100 and PM-Scl-75 [RNA processing and degradation]	**Diffuse SSc;** severe RP, arthritis, PF, calcinosis, myositis. In **Limited SSc** associated with myositis overlap. [211,212]
Anti-U3-snoRNP (anti-fibrillarin)	Fibrillarin and related U3 RNA components [pre-ribosomal RNA processing]	**Diffuse or limited SSc**; cardiac involvement, SRC, gastrointestinal involvement, ILD, PAH. [203,213-215]
Anti-U1-snRNP	U1 RNA and related components [mRNA splicing]	**Diffuse or limited SSc**; RP, MCTD (inflam-matory arthritis, myositis), PF, PAH. [27,28]
Anti-U11/U12 RNP	U11/U12 RNA and related components [mRNA splicing]	**Diffuse or limited SSc**; ILD

Table 5. Examples of functional (putatively pathogenic) auto-antibodies in SSc.

Autoantibody	**Functional effects**
Anti-endothelial cell	Induce endothelial cell apoptosis; finger ischemia, PAH [65,82,216]
Anti-fibroblast	Stimulate fibroblasts to produce ICAM-1, IL-6, contributing to vascular injury and ECM accumulation [65]
Anti-MMP-1 and anti-MMP-3	Prevent ECM degradation [217,218]
Anti-PDGF Receptor	Stimulate PDGFR signalling in fibroblasts, induce collagen I production, activate fibroblasts [81]
M3 muscarinic receptor	Stimulate receptor signalling in lower GI, inhibit muscle contraction [219]
Anti-AT_1R and anti-ET_AR	Stimulate AT_1R and ET_AR signaling, associated with PAH, SRC, DUs [220]
Anti-fibrillin-1	Fibroblast activation, ECM accumulation, TGFβ signalling [221]

1.2.4. Fibrosis in SSc

Fibrosis has long been considered *the* identifying feature of SSc, and a major component of SSc morbidity and mortality. Unlike most fibrotic diseases in which a single target organ is affected, SSc patients suffer from fibrosis of multiple vital organs throughout the body. With chronic fibrosis, there is a continuous accumulation of stiff connective tissue, which disrupts the affected organ's structure, thus causing its dysfunction and eventual failure. For researchers, SSc is considered to be a “model” fibrotic disease. Not surprisingly, this aspect of SSc pathology has received the majority of attention.

In the skin, fibrosis is ubiquitous, such that the terms SSc and scleroderma are often used interchangeably. Excessive amounts of various types of collagens and other ECM macromolecules are present in fibrotic SSc skin, as are myofibroblasts. Hair follicles and

sweat glands are impaired, if not completely obliterated by excessive ECM accumulation[222]. Esophageal dysfunction is a common symptom of SSc, resulting from fibrosis in the esophageal lamina propria, submucosa and muscular layers[223]. Myocardial fibrosis in SSc is distinctly different from fibrosis due to coronary artery disease, often occurring in a "mosaic" pattern[224] and has been reported in 50 to 80% of the SSc population[225]. Fibrosis of the lung, or interstitial lung disease, is a major cause of death in SSc[226], and affects 53% of dcSSc patients and 35% of those with the lcSSc subtype[226].

Tissue fibrosis can be considered to be a wound healing response that has failed to terminate. Wound healing is regulated and self-limited by a balance of pro- and anti-fibrotic cytokines and growth factors. In the context of fibrosis, fibroblast activation is uncontrolled, and by consequence, so are the proinflammatory, biosynthetic and contractile functions of these cells[227]. Fibroblasts are activated in response to various cellular signals. In pathological fibrosis, as opposed to the physiological process of wound healing, the fibrotic activity becomes amplified in a feed-forward cycle. Excessive ECM accumulation causes hypoxia and oxidative stress, increased tissue stiffness, and other signals, which stimulate further activation of fibroblasts.

1.2.4.1. Fibroblasts and Myofibroblasts

Fibroblasts are spindle-shaped cells of mesenchymal origin that constitute the main cellular component of the connective tissue and synthesize the interstitial connective tissue proteins throughout the body[228]. Fibroblasts are also the main cellular players in wound healing and fibrosis. Quiescent fibroblasts become activated by soluble mediators secreted by ECs, platelets, inflammatory cells and epithelial cells, and differentiate into myofibroblasts[229].

Unlike unstimulated quiescent fibroblasts, myofibroblasts secrete exaggerated amounts of ECM macromolecules, cytokines, growth factors and chemokines[230]. Myofibroblasts also migrate, adhere and contract ECM, which is necessary for wound closure[230]. TGF-β1 is a potent inducer of myofibroblast differentiation, as are ET-1[231,232], PDGF and FGF2[233], among others. ROS, hypoxia and mechanical signals from the ECM also stimulate myofibroblast differentiation[234]. Unlike quiescent fibroblasts, myofibroblasts express contractile proteins, like α-smooth muscle actin (α-SMA), which is widely used as a marker for distinguishing the two phenotypes. Myofibroblasts also constitutively synthesize and secrete numerous chemokines, cytokines and cell surface receptors, and are resistant to apoptosis[63,233,235].

Normally, α-SMA-positive myofibroblasts are present in early granulation tissue and are cleared by apoptosis, as the wound-healing process is resolved[236]. In fibrotic tissue, however, myofibroblasts persist, and maintain the excessive deposition of ECM components. The accumulation of collagens and other connective tissue proteins and the excessive contraction of the ECM by the myofibroblasts disrupt the normal tissue structure. The result is loss of function of the fibrotic organ[237].

Impaired degradation and ECM turnover is also an important facet of fibrosis. For example, myofibroblasts also synthesize tissue inhibitors of metalloproteinases (TIMPs)[238], which inhibit ECM degradation, thus further maintaining the excessive ECM characteristic of fibrosis. Accordingly TIMP-1 levels are elevated in SSc[238,239].

The fibroblast (and myofibroblast) population may be expanded by the differentiation of other cell types. Pericytes are contractile cells present in the basement membrane of the capillaries and small blood vessels[240,241], important for the regulation of microvascular circulation and permeability[242]. They have the potential to differentiate into fibroblasts and

myofibroblasts[243], providing a source of additional fibrotic cells[244], and thus a relationship between fibrosis and small blood vessel pathology. In support of this, reduced pericytes have been reported in SSc skin capillaries[245], suggesting migration and/or differentiation into fibroblasts. Similarly, a study of dermal fibrosis in SSc found that SSc skin biopsies included myofibroblasts while normal skin did not, and pericytes and myofibroblasts were found to express two myofibroblast differentiation markers not present in controls[244], implying that myofibroblasts may derive from pericytes in skin fibrosis[244]. Additionally, in SSc pericytes express receptors for the profibrotic growth factor PDGF[246].

Epithelial and endothelial cells may be transformed into fibroblasts as well, in processes called the "epithelial-mesenchymal transition" (EMT) and "endothelial-mesenchymal transition" (EndoMT). These mechanisms, both triggered by TGF-β, are thought to have a role in SSc fibrosis[247]. In particular, EndoMT transition may contribute to subendothelial accumulation of myofibroblasts and fibrotic tissue, leading to a fibroproliferative vasculopathy[248,249].

1.2.4.2. Transforming Growth Factor-β (TGF-β).

TGF-β is a master regulator of fibrosis. This pleiotropic growth factor has important roles in tissue repair processes and fibrotic disorders, immune regulation, the resolution of inflammation, and in embryonic development[250]. As mentioned, TGF-β stimulates fibroblast activation and drives ECM production. It also acts as a chemoattractant for fibroblasts. In the early phase of chronic inflammation, TGF-β is important for attracting monocytes and other leukocytes[251], while it also has immunosuppressive effects, inhibiting proliferation and activation of NK and T cells[251].

The three TGF-β isoforms, TGF-β1, TGF-β2, TGF-β3, are structurally almost identical but have independent and non-redundant roles *in vivo*[252]. TGF-β1 is the prototype and best understood member of the TGF-β family. These growth factors are secreted by most cells as homodimers kept inactive by a covalently bound latency associated peptide (LAP) and sequestered in connective tissue[252]. Cleavage of the LAP and release from the ECM are required for TGF-β to bind to its cognate receptor. There are type I and type II TGF-β receptors, coexpressed by most types of cells. TGF-β binds to its type II TGF-β receptor (ALK5), with its constitutively active serine-threonine kinase domain, which phosphorylates and forms an active complex with TGF-βRI (TGF-β receptor type I). The TGF-βRI phosphorylates Smad2/3, which ultimately forms a transcription factor complex and induction of target genes, such as type I collagens, α-SMA, and connective tissue growth factor (CTGF), to name but a few. TGF-β may also signal through alternative non-Smad pathways. In addition to stimulating collagen synthesis and fibroblast proliferation, TGF-β's profibrotis effects include inducing migration, myofibroblast transdifferentiation, ROS generation, fibroblast transdifferentiation via EMT and EndoMT, and downregulation of metalloproteinase expression[253].

Evidently, the TGF-β pathway has been heavily investigated for its roles in SSc. SSc tissue has been shown to have elevated TGF-β levels[254,255], although this is suspected to be caused by upstream activity. Both types of TGF-β receptors, are also upregulated in SSc. TGF-β, itself, as well as PDGF and EGF, stimulate TGF-β receptor expression. Because of its potent profibrotic activity, the TGF-β pathway is a logical pharmacologic target for SSc treatment. Blocking of the ligand, preventing activation of the latent form of TGF-β, and inhibiting receptor activity are some approaches that have been tested. The use of imatinib

mesylate, a tyrosine kinase inhibitor that also inhibits TGF-βR activity, in pre-clinical trials has shown promise[256-259], although clinically imatinib causes adverse effects of poor to acceptable tolerability. Presently, studies using nanoparticle delivery systems are ongoing, which may improve targeting of the drug and reduce side-effects[260,261].

1.2.4.3. Platelet-Derived Growth Factor (PDGF) and its Receptors

Platelet-derived growth factors are homo- and hetero-dimeric proteins secreted by platelets and many other cell types, including fibroblasts, endothelial cells, and macrophages. PDGFs have essential roles in embryonic development, vascular system physiology and wound healing, among others, and are heavily implicated in fibrosis and cancer. PDGF is a potent fibroblast chemoattractant and mitogen. There are four PDGF isoforms: PDGF-A, -B, -C, and -D; which form AA, BB, CC, and DD homodimers or the AB heterodimer[262]. These induce homo or heterodimerization of their cognate tyrosine kinase receptors PDGFRα and β. The AA, BB and AB forms of the ligand appear to be the most important in terms of fibrosis. PDGF-AA stimulates specifically PDGFRαα homodimers only, PDGF-AB will stimulate the αα and αβ receptor dimers, while PDGF-BB has high affinity for all three (αα, αβ, and ββ). Ligand binding will induce receptor dimerization and subsequent autophosphorylation of specific tyrosine residues in the intracellular domain, which then activate signaling pathways that include the PI3K/Akt, PLC-γ1/PKC, Src, and Ras-Raf-ERK1/2 pathways[263], leading to cellular proliferation, chemotaxis, and actin reorganization[263-266].

Interestingly, a small proportion of PDGFRs will constitutively associate with the epidermal growth factor receptor (EGFR) in cell types expressing both receptors[267]. These receptor heterodimers have been shown to form heterodimeric complexes in response to ligand

binding[268]. In these cases, the receptors may facilitate signaling pathway activation of each others' canonic (even if overlapping) signaling cascades[267,268].

PDGF and its receptors' signal transduction pathways constitute another rational pharmacotherapeutic target for SSc. Both the ligand and its receptors are elevated in SSc endothelium and capillaries[246,269-271], in lung[272], and in SSc skin. In addition, TGF-β induces PDGFR expression in SSc skin[269,271], and lung fibroblasts[271,272], thus increasing fibroblast responsiveness to PDGF. Similarly, SSc fibroblasts have been reported to secrete increased amounts of IL-1α, which is also a stimulant for PDGF production[273]. Autoantibodies that target and have agonistic activity on PDGFR have also been reported, leading to the stimulation of ROS and MAPK signaling and fibroblast activation[81]. Thus, there are various factors which may contribute to persistent PDGF signaling in SSc cells, leading to excessive secretion of ECM components (collagen, fibronectin, proteoglycans) and also of soluble mediators TGF-β1, MCP-1 and IL-6[274].

Small-molecule inhibitors of PDGF tyrosine kinase activity may improve SSc skin fibrosis[275]. The tyrosine kinase inhibitor imatinib mesylate blocks PDGFRs and also related proteins c-Kit and c-Abl. c-Abl is also a critical component of TGFβ signal transduction, allowing imatinib the possibility of dual inhibition of two profibrotic signals at once. Newer tyrosine kinase inhibitors are also being studied as potential SSc therapy. Sunitinib has been demonstrated to prevent SSc-like disease development induced chemically in mice[276]. The use of monoclonal antibodies is also being investigated, and seems promising in preclinical models[277,278].

1.2.4.4. Epidermal Growth Factor (EGF) and its Receptors

The Epidermal Growth Factor Receptors (also called ErbB), which include EGFR (also called ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4)) were once deemed the prototypical tyrosine kinase receptors, but are now known to have unique features. EGFRs are activated upon binding of ligands from the epidermal growth factor (EGF) family, which include EGF, TGFα, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epigen, and epiregulin[279,280]. The EGFR ligands are expressed as transmembrane proteins, which undergo proteolytic cleavage by membrane-bound metalloproteinases (such ADAM17 (a disintegrin and metalloproteinase 17)) in a process termed ectodomain shedding. The released EGF-like domain may then stimulate its cognate receptors in an autocrine, paracrine and/or endocrine fashion[279,280]. Like PDGFR, dimerization of these tyrosine kinase receptors brings enzymatically active domains in proximity with their target tyrosine sites. Phosphotyrosine residues then initiate various signaling cascades, including RAS/MAPK, PI(3)K/Akt, PLCγ1/PKC and STAT, signal transduction pathways[281].

Interestingly, of the four EGFRs only EGFR, ErbB2 and ErbB4 are kinase active. While ErbB3's intracellular domains have no tyrosine kinase activity, it will dimerize with the other EGFRs, thereby allowing it signal transducing activity[282]. ErbB2, on the other hand, does not bind to any known ligands, but dimerizes readily with the other EGFR types[282,283]. Thus, in addition to ligand specificity (and other mechanisms beyond the scope of this thesis), dimerization of different EGFR partner combinations is an important mechanism regulating signal transduction activity.

EGFR signaling is important in early development and growth, and it is overexpressed in many tumours. Its role in cancer has been extensively explored, and accordingly, a number

of EGFR-inhibiting molecules are used as anti-cancer drugs. The importance of EGFR signaling in SSc is less well-known. A 1990 investigation of fibroblasts from 3 SSc patients reported reduced EGFR-EGF affinity[284]. More recently, Planque *et al.* (2012)[285] found that some SSc (and SLE) patients possess autoantibodies that bind to a recombinant extracellular domain of EGFR, and these autoantibodies inhibit DNA synthesis in cultured EGFR-expressing cells. However the significance of these findings remain to be explored further.

1.3. Animal Models

A number of animal models of SSc have been discovered, all of which express a fibrotic phenotype[286-288]. Although these are of key importance in SSc research, particularly given the scarcity of human samples available for laboratory testing, the majority of these models tend to recapitulate only partially the spectrum of human SSc features. To date, only two SSc animal models present the fibrotic, vascular, inflammatory *and* autoimmune characteristics. Evidently, the molecular and phenotypic differences among the various animal models of SSc have led to insights into molecular mechanisms underlying features of SSc pathopysiology.

One of the most commonly used model is bleomycin-induced fibrosis in mice. Mice receiving subcutaneous bleomycin injections manifest skin and lung fibrosis that resemble biochemically (presence of TGF-β and ANAs) and histologically (mast cell infiltration, collagen accumulation) those features in SSc. However, fibrosis of other organs and vasculopathic changes do not occur[289].

The tight skin mouse (TSK-1) is the most commonly used and best characterized non-inducible SSc model. It is a spontaneous mutant with fibrosis as the predominant feature.

Although activated B cells and autoantibodies are present in this model, inflammatory processes and SSc-like vasculopathy are absent.

The Fra-2 transgenic mouse model, on the other hand, displays SSc-like vascular features (EC apoptosis and disappearing capillaries) and inflammation that precede significant skin and organ fibrosis, but no signs of autoimmunity. It has been deemed a useful model for studying the development of PAH and interstitial lung disease[290,291]. Fos-related antigen 2 (Fra-2) is overexpressed in the tissues of these mice. Fra-2 is a component of the Fos/AP-1 transcription factor. The generation of Fra-2 ectopically expressing mice revealed a key role for this protein in fibrogenesis[291]; accordingly, Fra-2 is upregulated in SSc patients[292].

Perhaps the most comprehensive animal models for SSc, UCD 200/206 chickens closely mirror human serological, histopathological and clinical SSc features[293-295]. This spontaneous avian model exhibit anti-endothelial cell antibodies that induce apoptosis early in the disease's progression[296]. Unfortunately, they are less practical to use than mice, considering their long generation time and the paucity of biological data related to their use as human disease models, as well as the limited availability of reagents for use in chickens.

Recently, a new mouse model doubly heterozygous for Fli1 and Klf5 genes have has been generated[297]. Krüppel-like factor 5 (Klf5) and Friend leukemia virus integration 1 (Fli1) are transcription factors that are epigenetically suppressed in SSc[298,299], and that have roles in modulating fibrosis. The $Klf5^{+/-}$; $Fli1^{+/-}$ mice manifest the full spectrum of SSc features spontaneously, inflammation and small vessel vasculopathy, fibrosis of the skin and lung, upregulation of CD19 and accordingly B cell activation, and autoantibody production. This model suggests that the epigenetic suppression of these transcription factors may be central to SSc pathology[297].

Chapter 2. Published Original Manuscript

Systemic sclerosis immunoglobulin induces growth and a pro-fibrotic state in vascular smooth muscle cells through the epidermal growth factor receptor

2.1. Rationale, Hypothesis, and Objectives

Systemic sclerosis (SSc) is an autoimmune rheumatic connective tissue disease, characterized by uncontrolled fibrosis of the skin and internal organs, specific autoantibody profiles, and vascular manifestations which include Raynaud's phenomenon, pulmonary arterial hypertension, endothelial dysfunction, and other vascular abnormalities.

In 2006, Baroni and colleagues found that all SSc patients in their cohort possessed agonistic autoantibodies directed to the platelet-derived growth factor receptor (PDGFR) on fibroblasts, leading to changes in cell signaling events, and culminating in increased collagen gene expression. In addition, these autoantibodies were absent from all non-SSc patients[81]. This provided a direct link between the autoimmune and fibrotic disease manifestations that define SSc, and also stimulated further investigations into the etiological role of this mechanism. However, these findings are now considered controversial, with several other research groups unable to reproduce these findings in their SSc cohorts[197-199].

The purpose of this project was to explore the effects of purified SSc IgG on vascular smooth muscle cells (VSMCs), given the importance of vascular abnormalities in SSc. We hypothesized that if these autoantibodies do indeed exist, then they are likely to come into contact with and stimulate the PDGFR on VSMCs as well, and this may relate to vascular abnormalities in SSc. Since Riemekasten *et al.*[220] have reported autoantibodies that activate AT_1R on endothelial cells, we hypothesized that SSc IgG may also activate the AT_1R in VSMCs. Finally, we did not hypothesize that the EGFR would be stimulated by SSc IgG, as others have excluded its involvement with the use of EGFR inhibitors[81]. However, since it is known to interact with AT1R and with PDGFR we intended to demonstrate its lack of involvement in SSc-IgG stimulated signaling in VSMCs.

Our objectives were:

1. to validate the presence of PDGFR-antibodies in SSc sera by measuring ERK phosphorylation in VSMCs exposed to purified IgG, and by using pharmacological inhibitors to block PDGFR signaling in these cells;
2. to characterize the response of VSMC to SSc IgG by measuring PDGFR binding, ERK and Akt phosphorylation, collagen and TGF-β gene expression, DNA and protein synthesis;
3. to investigate whether the EGFR and AT1R might play a role in VSMC stimulation by SSc IgG.

2.2 Author Contributions

Monique Arts is the first author. She designed, carried out the experiments, compiled and analyzed the data, and rendered all figures and tables used to present data and ideas. She wrote and edited the manuscript. Dr. Murray Baron is the director of the Canadian Scleroderma Research Group, and provided access to the patient samples and any affiliated relevant patient information used in this study. Serum samples were obtained by rheumatologists participating in the CSRG across Canada, and were stored and maintained at a central CSRG location at the Lady Davis Institute in Montreal. Dr. Baron also contributed to the writing of the manuscript. Nidaa Chokr carried out some immunoprecipitation experiments, prepared the material for qRT-PCR analysis, and contributed to the analyses of these data. The Canadian Scleroderma Research Group consists of the rheumatologists across Canada who recruited patients and obtained samples and data for the CSRG biobank, from which we obtained our samples (membership of the CSRG is provided in the Acknowledgments of the manuscript (section 2.9.1)). All members acknowledged the manuscript and gave their agreement for its submission for publication. Marc J. Servant is the lead author. He provided important guidance in the design and performance of the experiments, the analysis of the data, and the writing of the manuscript.

2.3 Title Page

Systemic sclerosis immunoglobulin induces growth and a pro-fibrotic state in vascular smooth muscle cells through the epidermal growth factor receptor

AUTHORS

Monique R. Arts[1,2], Murray Baron[2], Nidaa Chokr[1], Marvin J. Fritzler[3], the Canadian Scleroderma Research Group (CSRG)[†], and Marc J. Servant[1*]

*corresponding author contact information: marc.servant@umontreal.ca

[†]membership of the CSRG is provided in the Acknowledgments

AFFILIATIONS

[1]Université de Montréal, Faculté de Pharmacie, Montreal, Quebec, Canada; [2]McGill University, Jewish General Hospital, Montreal, Quebec, Canada; [3]Department of Medicine, University of Calgary, Calgary, Alberta, Canada.

PUBLISHED:

June 13, 2014: PLoS ONE 9(6): e100035

https://doi.org/10.1371/journal.pone.0100035

2.4 Abstract

Objective. It has been suggested that autoantibodies in systemic sclerosis (SSc) may induce the differentiation of cultured fibroblasts into myofibroblasts through platelet-derived growth factor receptor (PDGFR) activation. The present study aims to characterize the effects of SSc IgG on vascular smooth muscle cells (VSMCs) and to determine if stimulatory autoantibodies directed to the PDGFR can be detected, and whether they induce a profibrotic response in primary cultured VSMCs.

Methods. Cultured VSMCs were exposed to IgG fractions purified from SSc-patient or control sera. VSMC responses were then analyzed for ERK1/2 and Akt phosphorylation, PDGFR immunoprecipitation, cellular proliferation, protein synthesis, and pro-fibrotic changes in mRNA expression.

Results. Stimulatory activity in IgG fractions was more prevalent and intense in the SSc samples. SSc IgG immunoprecipitated the PDGFR with greater avidity than control IgG. Interestingly, activation of downstream signaling events (e.g. Akt, ERK1/2) was independent of PDGFR activity, but required functional EGFR. We also detected increased protein synthesis in response to SSc IgG ($p<0.001$) and pro-fibrotic changes in gene expression (*Tgfb1* +200%; *Tgfb2* -23%; $p<0.001$)) in VSMCs treated with SSc IgG.

Conclusion. When compared to control IgG, SSc IgG have a higher stimulation index in VSMCs. Although SSc IgG interact with the PDGFR, the observed remodeling signaling events occur through the EGFR in VSMC. Our data thus favour a model of transactivation of the EGFR by SSc-derived PDGFR autoantibodies and suggest the use of EGFR inhibitors in future target identification studies in the field of SSc.

2.5 Introduction

Systemic sclerosis (SSc) is a chronic disorder of connective tissue characterized by autoimmunity, inflammation, fibrosis and vascular disease. Although the aetiology of this disease is poorly understood, possible contributory factors include agonistic autoantibodies that target receptors, which in turn may contribute to the SSc pathology. One such autoantibody has been reported to target and activate the platelet-derived growth factor receptor (PDGFR) on fibroblast cells, leading to downstream signaling activity and culminating in pro-fibrotic events [1]. Such autoantibodies were not detected in any controls but were in all SSc patients tested and also in graft-versus-host disease [2], two conditions characterized by fibrosis and autoimmunity. The B-cell depleting drug Rituximab, which may have beneficial effects on lung function and skin fibrosis in SSc patients [3], has been associated with reduced PDGFR phosphorylation in SSc skin [3], supporting the possibility of agonistic anti-PDGFR-autoantibodies. In contrast, others have reported that both healthy and SSc patients possess such autoantibodies, and/or that such autoantibodies do not lead to signaling activity [4-6]. PDGFR-activating auto-antibodies have also been reported in systemic lupus erythematosus (SLE) patients [7].

Proliferative and obstructive vasculopathy is common in SSc. Most patients experience Raynaud's phenomenon, and many develop vascular ulcers of the extremities [8]. Pulmonary arterial hypertension (PAH) in SSc is a leading cause of mortality and affects 10-12% of patients [9]. PAH is characterized by increased pulmonary vascular resistance and features uncontrolled endothelial and vascular smooth muscle cell (VSMC) growth, vasoconstriction and extracellular matrix accumulation, thus obstructing the pulmonary arterial circulation [9,10]. VSMCs are known to express PDGFRs and in these cells PDGF is a mitogen [11].

Moreover, PDGFR-β immunoreactivity was reported to be more prevalent and intense in the pulmonary vessels of a SSc-PAH group than in controls [12]. Thus, if PDGFRs on VSMCs are activated by anti-PDGFR autoantibodies present in the circulation of SSc patients, this could contribute to the ubiquitous vascular pathology of the disease. We therefore sought to determine if SSc sera contain antibodies that stimulate VSMCs and if so, whether these antibodies are directed to PDGF receptors.

It is also noteworthy that the expression levels of both the PDGFR and epidermal growth factor receptor (EGFR) are significantly increased in the vasculature of SSc-PAH patients compared to controls [12]. In a molecular context, a certain proportion of these receptors are expressed as heterodimers in VSMCs and interestingly, the activation of early signaling events by PDGF in these cells occurs through transactivation of the EGFR via a process that is independent of PDGFR kinase activity but dependent on EGFR kinase activity [13,14]. Thus, we investigated whether the EGFR might play a role in VSMC stimulation by SSc IgG. It is also known that Angiotensin II acts as a growth factor and pro-inflammatory cytokine for cultured VSMCs through binding to the Angiotensin II Type I receptor (AT1R) [15,16]; thus this G protein-coupled receptor is recognized as a major vascular remodeling effector [17]. Interestingly, transactivation of the PDGFR by the AT1R has been shown to be involved in VSMC signaling events [18]. Moreover, activating anti-AT1R autoantibodies have been reported in SSc [19]. We therefore also investigated the possibility that SSc IgG may stimulate VSMCs through the AT1R.

2.6 Patients and Methods

2.6.1 Ethics Statement.

All serum samples were obtained from patients or control subjects who had provided written informed consent for the use of their biological samples. The study was approved by the ethics committee of the Université de Montréal (CERSS#919) and by the ethics board of the SMBD Jewish General Hospital, Montreal, Quebec, Canada. Our use of animal-derived cells was approved by the Animal Ethics Committee of the Université de Montréal (protocol #09-156) and conformed to the Guide for the Care and Use of Laboratory Animals.

2.6.2 Patients and biological samples.

Serum samples from 23 SSc patients were obtained from the biobank of the Canadian Scleroderma Research Group (CSRG), maintained at the University of Calgary. For this study we required that subjects have early (5 years or less since onset of first non-Raynaud's symptom) diffuse cutaneous SSc according to 1980 ACR preliminary classification criteria [20], and not be on any immunosuppressive or steroid therapy. Prospective data collected on each patient by their rheumatologists at the time of the patient's visit, as previously described [8], included: Modified Rodnan skin score [21], Medsger vascular disease severity [22], presence of any active vascular cutaneous ulcers, pulmonary hypertension (PH; defined as an estimated systolic pulmonary arterial pressure (PAP) $\geq$ 45 mmHg on echocardiogram which correlates strongly with right heart catheter studies [23,24]), and presence of anti-centromere, anti-topoisomerase, and anti-RNA Polymerase III antibodies. Anti-centromere antibodies were detected by indirect immunofluorescence staining of HEp-2 cell substrates (ImmunoConcepts Inc., Sacramento, CA), while anti-topoisomerase antibodies were measured by an addressable

laser bead immunoassay using an INOVA ENA 9 QuantaPlex kit (INOVA Diagnostics, San Diego, CA) and a Luminex 100 illuminometer (Luminex Corp., Austin, TX), and antibodies to RNA Polymerase III were detected by ELISA (INOVA) [25].

Controls from CSRG-participating clinics consisted of five age- and sex-matched otherwise healthy individuals with osteoarthritis who were not on corticosteroids or immunosuppressives, seven normal healthy individuals, one SLE patient and one sample from a normal pooled blood bank.

2.6.3 Immunoglobulin purification.

IgG was purified from serum using Immobilized Protein A/G (Pierce, Rockford, IL) according to manufacturer recommendations, scaled down for use with Handee Mini Spin Columns (Pierce). All binding, wash and elution steps were performed by gravity-flow. The flow-through was reapplied three times, and the bound protein washed three times with 1M NaCl and 8 times with binding buffer (Pierce). Eluted IgG was immediately neutralized with Tris-HCl pH8.5 and concentrated using Amicon 100 kDa centrifugation tubes (Millipore, Billerica, MA) according to manufacturer instructions, thereby excluding any growth factors or other molecules smaller than 100 kDa. To verify the efficiency of IgG purification and the quality of purified IgG samples, eluates were examined by 10% acrylamide SDS-PAGE followed by Coomassie blue staining. A bicinchoninic acid (BCA) assay (Pierce) was used to determine concentration of IgG samples. Samples were aliquoted and stored at -80˚C until required for use in cell stimulation experiments.

2.6.4 Reagents, antibodies and pharmacological inhibitors.

Recombinant human PDGF-BB and EGF were obtained from Biosource (Camarillo, CA). Recombinant rat PDGF-BB and recombinant human TGF-β1 were purchased from R&D Systems (Minneapolis, MN). Angiotensin II was from Sigma (St. Louis, MO). The following pharmacological inhibitors were used in our study: PDGFR inhibitors AG1296 (Calbiochem, Gibbstown, NJ) and imatinib mesylate (Alexis Biochemicals, San Diego, CA); AT1R inhibitor irbesartan (a kind gift from Dr. Pierre Moreau, Université de Montréal); and EGFR inhibitor AG1478 (Biomol, Plymouth Meeting, PA).

Antibodies specific for Phospho-p44/42 MAPK (pERK1/2) (Thr202-Tyr204) and p44/42 MAP Kinase (ERK1/2), Phospho-Akt (Ser473), Akt, Phospho-EGF Receptor (Tyr845) and EGF Receptor (C74B9) were from Cell Signaling Technology (Beverly, MA). Anti-Mouse CD140a (PDGFR a) and CD140b (PDGFR b) Functional Grade Purified neutralizing antibodies were obtained from eBioscience (San Diego, CA). Anti-PDGFRα and β antibodies were from Upstate Biotechnology (Lake Placid, NY).

2.6.5 Cell culture.

Aortic VSMCs were isolated from Wistar rats by explant and maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS). MRC5 cells were obtained from ATCC, and cultured according to supplier recommendations. Cells at 75% confluence were rendered quiescent by incubation for 48 hours in serum-free high glucose DMEM and Ham's F-12 (1:1) supplemented with 15 mM Hepes (pH 7.4), 0.1% low-endotoxin bovine serum albumin (Sigma), and 5 μg/mL transferrin (Sigma) for 48 h. Experiments were conducted on cells at passages 5-13. For experiments with pharmacological inhibitors, cells

were pre-treated for 30 minutes with vehicle alone or with the indicated concentrations of inhibitors.

Cells were maintained in the absence of antibiotics and routinely tested for mycoplasma contamination using LookOut™ Mycoplasma PCR Detection Kit (Sigma) or MycoAlert™ Mycoplasma Detection Kit (Lonza, Rockland, ME).

2.6.6 Immunoblot analyses.

350,000 VSMCs were seeded in 6-well plates in DMEM/10%FBS. Quiescent cells were stimulated with 200 μg/mL purified IgG [1] for 5 minutes, at 37°C under 5% CO_2. After 5 minutes, cells were gently washed two times with ice-cold PBS and whole cell extracts were prepared using Triton-X lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, 1 μM pepstatin A, 2 μg/mL aprotinin, 1% Triton-X-100, 10% glycerol) for 30 min at 4˚C. Lysed material was then centrifuged at 13,000 x *g* for 10 min and the supernatant collected. Equal amounts of lysate proteins (20-50 μg) were loaded on 7.5 or 10% polyacrylamide gels and subjected to SDS-PAGE. In some Western blot experiments, cellular extracts were divided and used in parallel. Proteins were transferred to nitrocellulose membranes in 25 mM Tris, 192 mM glycine and 20% methanol using a Bio-Rad Transblot Cell transfer apparatus. Immunoblotting with each antibody was carried out according to manufacturer instructions.

Western blot bands were analyzed by densitometry using ImageQuant TL version 2002 (Amersham, NJ). Densities of p-ERK bands were normalized to corresponding total ERK bands. A stimulation index was determined for each sample using the equation (S-C)/(P-C) x

100 where S, C, and P represent the normalized band intensities of a given sample, the negative control and the positive control, respectively [1]. In addition, we measured the phosphorylation of ERK2 by ELISA, using DuoSet® IC: Human/Mouse/Rat Phospho-ERK2 (T185/Y187) ELISA kit (R&D Systems) according to manufacturer recommendations.

2.6.7 Immunoprecipitation assays.

Quiescent VSMCs were lysed on ice, using a RIPA buffer (50 mM Tris-HCl (pH7.4 at 4˚C), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, 1% Triton-X-100, 10% glycerol, 0.1% SDS, and 1% Na-deoxycholate, 1 mM sodium orthovanadate, 1 μg/mL pepstatin, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 0.2 mM PMSF) for 30 minutes. 500 μg of whole cell extracts were incubated for 4 hours at 4˚C with 2 μg of anti-PDGFR-β (Upstate), 200 μg SSc or control IgG immobilized on 50 μL protein-A-Sepharose beads (GE Healthcare). The immune complexes were washed four times with Triton X-100 lysis buffer and 2X Laemmli's sample buffer was added. The immunoprecipitated proteins were analysed by immunoblotting using commercial anti-PDGFRβ antibodies.

2.6.8 RT-qPCR analysis.

150,000 VSMCs were seeded in 6-well plates in DMEM/10%FBS. Quiescent cells were stimulated with 200 μg/mL purified IgG [1] for 2 hours (*col1a1* and *colIII*) or 72 hours (*Tgfb1*, *Tgfb2*, *Tgfb3*), at 37°C under 5% CO_2. Cells were then gently washed with ice-cold PBS and flash-frozen in liquid nitrogen. Next, cell lysates were collected and total RNA isolated using RNeasy Mini Kit (QIAGEN) according to manufacturer directions, and spectrophotometric quantification of RNA samples was performed. RNA integrity was verified using a Bioanalyzer system at the Institute for Research in Immunology and Cancer

(IRIC; Montreal). Total RNA (2 μg) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit with random primers (Applied Biosystems) as described by the manufacturer. Real-time PCRs were subsequently performed using the Fast SYBR Green Master Mix (Applied Biosystems) with the following primers: ***colIII***: FWD 5'-AGATGCTGGTGCTGAGAAG-3'; REV 5'-TGGAAAGAAGTCTGAGGAAGG-3'; ***Tgfb1***: FWD 5'-CCTGGAAAGGGCTCAACAC-3'; REV 5'-CAGTTCTTCTCTGTGGAGCTGA-3'; ***Tgfb2***: FWD 5'-AGTGGGCAGCTTTTGCTC-3'; REV 5'-GTAGAAAGTGGGCGGGATG-3'; ***Tgfb3***: FWD 5'-AGTGGCTGTTGCGGAGAG-3'; REV 5'-GCTGAAAGGTATGACATGGACA-3'; ***Actb***: FWD 5'-CCCGCGAGTACAACCTTCT-3'; REV 5'-CGTCATCCATGGCGAACT-3' (UPL probe #17). Quantitect primer assay (QIAGEN) was used for ***col1a1***. β-actin was used as endogenous control. Samples were initially denatured at 95˚C for 3 min followed by 40 cycles of 5 sec at 95˚C and 30 sec at 60˚C. All reactions were run in triplicate and the average threshold cycle (Ct) values were used for quantification. The relative quantification of target genes was determined using the DDCT method [26]. Briefly, the Ct values of target genes were normalized to those of an endogenous control gene, β-actin ($DCt = Ct_{target} - Ct_{CTRL}$) and compared with a calibrator: $DDCT = DCt_{Sample} - DCt_{Calibrator}$. Relative expression (RQ) was calculated as $RQ = 2^{-DDCT}$.

2.6.9 [^{3}H]-leucine and [^{3}H]-thymidine incorporation assays.

45,000 or 60,000 VSMCs were seeded per well in 24-well plates for [^{3}H]-leucine and [^{3}H]-thymidine incorporation assays, respectively. After 24 hours, cells were rendered quiescent for 48 hours, then stimulated in triplicate with positive controls or purified IgG samples (200 μg/mL). For cell proliferation assays, [^{3}H]-thymidine (MP Biomedicals) was

added to a final concentration of 0.5 μCi/mL, and for protein synthesis assays, [^{3}H]-leucine (MP Biomedicals) was added to a final concentration of 0.3 μCi/mL. Cells were then incubated for 24 hours at 37˚C, 5% CO_2. After 24 hours, media was removed, and cells were washed and fixed overnight at 4°C with ice-cold 5% tri-chloro-acetic acid (Fisher). Plates were gently washed with water and once dry, the cells were solubilized in 0.1N NaOH and added to scintillation tubes containing EcoLite(+) scintillation cocktail (MP Biomedicals), and vortexed vigorously. Radioactivity was measured using a Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard) as counts per minute (CPM) per well and expressed as fold change from basal.

2.6.10 Statistical analyses.

Statistical analyses were performed using GraphPad Prism version 5.0 for Mac (GraphPad Software, San Diego, CA). Comparison of two groups was carried out by two-tailed t-test, and comparison of more than two groups was carried out with one-way ANOVA and a Bonferroni post-test. Statistical significance was accepted at $P \leq 0.05$.

2.7 Results

2.7.1 Patients.

We studied sera from 23 SSc patients with a disease duration of 5 years or less since first non-Raynaud's symptom and not on any steroid or immunosuppressive therapy. General characteristics of our patient cohort are summarized in Table 1 (details of individual subject characteristics are available in Supplemental Table S1). 91% of patients were female. The mean age was 48.7 years. The mean modified Rodnan Skin Score was 16.5 and the mean

Medsger vascular disease severity score was 1.9. 36% of SSc patients had active vascular ulcers and 4.8% had pulmonary hypertension on echocardiography. 19% of patients had anti-centromere antibodies, and 29% had anti-topoisomerase antibodies. A large proportion, 43%, of our patients had anti-RNA polymerase III antibodies, which is not surprising since this antibody is more common in diffuse cutaneous SSc [25,27]. 8.7% of our patient group had received some immunosuppressive treatment less than a year prior to having their blood drawn. The 13 controls consisted of 5 normal subjects, 5 osteoarthritis patients, one SLE patient and one sample from a normal pooled blood bank. The mean age of controls was 43.6 (SD=16.5) and 83% were females.

2.7.2 Purified IgG from SSc sera have a greater stimulation index in VSMC than normal IgG.

Quiescent VSMCs were exposed for 5 minutes to 200 μg/mL IgG purified from SSc and control sera, and ERK1/2 and Akt phosphorylation were analyzed as an indication of activation of early signaling pathways involved in vascular remodeling events [28,29]. As previously conducted [1], a stimulation index was determined for each sample (Table S1) by densitometric analyses of Western blots (Fig. 1A). A comparison of the mean stimulation activity of SSc IgG with that of control IgG revealed a higher stimulation capacity in the SSc samples (p=0.0474; Fig. 1B), and the highest levels of ERK1/2 and Akt phosphorylation were observed in cells treated with SSc IgG (Fig. 1B).

In addition to immunoblot analyses, we measured by ELISA the ability of a subset of IgG samples (SSc subjects 3 and 6-15) to induce ERK2 phosphorylation. Again, the increase in ERK2 phosphorylation in SSc-IgG-treated VSMCs was greater than in control-IgG-treated

cells (Fig. 1C). Using sera from 2 SSc subjects, cells were exposed to 50, 100, 150 and 200 μg/mL IgG. The increase in ERK1/2 phosphorylation was dose-dependent (Supp. Fig. S1).

The purified IgG samples had the same effects on other cell lines. Although we studied the effects of SSc IgG mainly in VSMCs obtained from rat cells, the increased phosphorylations of Akt also occurred in cells of human origin. Primary diploid human fibroblasts (MRC5 cells) exposed to different SSc IgG fractions exhibited increased Akt phosphorylation, similar to that seen in rat VSMCs (Supp. Fig. S2).

2.7.3 PDGFR-β interacts more avidly with SSc IgG than control IgG but does not mediate the increase in ERK1/2 and Akt signaling.

In order to address the ability of SSc IgG to recognize and specifically bind the PDGFR, we next performed immunoprecipitation studies using purified IgG from a subset of 8 SSc and 7 control subjects (Representative data shown in Fig. 2A, B). Although there was variation among samples, on average, the SSc-IgG immunoprecipitated significantly more PDGFR-β than control IgG (p=0.0385; Fig. 2C). We did not observe any statistically significant correlation between the stimulation index of individual samples (Table S1) and the capacity to immunoprecipitate the PDGFR-β.

We next investigated whether the catalytic activity of the PDGFR was involved in SSc-IgG-induced early signaling events in VSMC. The quinoxaline AG1296 is a highly potent and selective inhibitor of the PDGFR-α and β isoforms and its family members c-kit and flt3 [30,31]. Cells pre-treated with AG1296 had indeed a dramatic reduction in ERK1/2 phosphorylation upon stimulation with PDGF-BB. However, SSc-IgG-induced ERK1/2 and Akt phospho-signals were not affected by the use of this PDGFR kinase inhibitor (Fig. 3A,

Supp Fig. S3). To further substantiate these observations, cells were also pre-treated with imatinib mesylate, which, in addition to blocking the kinase activity of c-Abl also antagonizes the PDGFR and c-kit [32]. Once again, the observed SSc-IgG-induced activation of ERK1/2 and Akt was not affected by this drug although it diminished the response to PDGF-BB stimulation (Fig. 3B, C). Thus, despite the ability of the SSc-IgG fractions to recognize and stably interact with the PDGFR-β, our data suggest that they do not use the kinase activity of this receptor family to transmit downstream intracellular signals.

2.7.4 Epidermal growth factor receptor (EGFR), but not the AT1R, plays a key role in the VSMC response to SSc IgG.

Since anti-AT1R autoantibodies have been demonstrated in SSc [19] we tested the AT1R antagonist irbesartan in SSc-IgG stimulated cells. In our study, the AT1R antagonist irbesartan abolished Ang II signaling in VSMC but did not affect SSc IgG-induced ERK and Akt phosphorylation (Fig. 3C). The potent and selective EGFR kinase inhibitor AG1478 [33] was also tested. At very low concentration, this compound significantly reduced EGF-induced phosphorylation of ERK1/2 and Akt (Fig. 3C). As previously shown [34], AG1478 also diminished the ability of Ang II to induce ERK1/2 and Akt activation resulting from AT1R-EGFR transactivation [18]. More importantly, blocking the EGFR phospho-transferase activity severely affected SSc IgG-induced activation of these early signaling events, suggesting a role for the EGFR's catalytic activity in the cellular response to SSc IgG (Fig. 3C).

2.7.5 SSc IgG causes growth and pro-fibrotic responses in VSMCs.

To address the potential of SSc IgG to cause pathophysiological changes in VSMCs that are linked to vascular remodeling events, we measured changes in cell growth and proliferation. These experiments were conducted with a subset of our SSc (n=10) and control IgG samples (n=5). Cell proliferation studies, as measured by incorporation of radiolabeled thymidine after incubation of VSMC with 200μg/mL IgG, indicated that neither SSc nor control IgG caused any detectable increase in DNA synthesis (data not shown). Purified IgG from both controls and SSc patients did cause an increase in protein synthesis as measured by radiolabeled leucine incorporation, but protein synthesis was greater in VSMCs stimulated with SSc IgG (Fig. 4A). While on average, control IgG increased protein synthesis by 46% above basal levels, SSc IgG caused a mean increase of 93% (p=0.0008).

Given the fibrotic features of SSc, we measured the expression of genes with roles in fibrosis, specifically *Col1a1*, *ColIII*, and *Tgfb1*, *2*, and *3*. Most importantly, we found that VSMCs responded to stimulation with purified SSc IgG by modulating the gene expression of TGF-β isoforms 1 and 2 (Fig. 4B, C). TGF-β is considered a master regulator of fibrotic processes [35]. Cells were stimulated with PDGF and TGF-β1, which modulates its own gene expression, as positive controls. Again, the IgG buffer solution alone had no detectable effect on the expression of any of the three protein isoforms. *Tgfb1* expression was induced significantly by all SSc IgG samples tested, and was induced to a lesser degree by some, but not all, control IgG samples (Fig. 4B). On average, SSc IgG caused a 2-fold induction of *Tgfb1* in VSMCs, which was significantly higher than control IgG (p<0.0001). In contrast, *Tgfb2* expression was decreased in cells treated with SSc IgG (p=0.0009); Fig. 4C). *Tgfb3* gene expression was not affected by treatment with SSc IgG (data not shown). Also, SSc IgG

did not affect expression of the collagen genes, *col1A1* or *colIII* in VSMCs after 72 h (data not shown), despite this being the time point at which PDGF and TGF-β caused the greatest modulations in *col1A1* or *colIII* gene expression in these cells (data not shown).

2.7.6 Relationship to disease phenotype.

Patients were grouped according to various disease manifestations (e.g. presence or absence of vascular ulcers, level of disease severity, mRSS, autoantibodies, pulmonary hypertension, etc.) and compared for differences in assay results (e.g. IgG stimulating activity, protein synthesis, etc), but no significant differences were found between groups (data not shown). Similarly, past use of immunosuppressive therapy did not have any effect on the cell-based assay results.

2.8 Discussion

Our study demonstrated that SSc IgG induced growth and profibrotic responses in cultured VSMCs, which are known contributors to obstructive vasculopathy. We found that exposure of VSMCs to SSc IgG led to activation of protein kinases known to regulate vascular remodeling events. Activation of PDGFR and/or EGFR leads to initiation of numerous signaling cascades, including the ERK1/2 and Akt pathways [36,37]. In VSMCs, ERK1/2 has been shown to be a crucial signaling molecule involved in various aspects of vascular remodelling, including the control of TGF-β gene expression, thus the fibrotic response, upon PDGF stimulation [28], and the protein kinase Akt regulates numerous cellular processes, including protein synthesis [38-40]. The increases in ERK1/2 and Akt phosphorylation that we observed were not inhibited by PDGFR inhibitors but were inhibited by an EGFR inhibitor, despite SSc IgG binding to PDGFRβ. Also, there was greater protein synthesis in VSMCs

stimulated with SSc IgG, as well as simultaneous *Tgfb1* upregulation and *Tgfb2* downregulation, in response to VSMC exposure to SSc IgG. TGF-β1 is generally considered to have important pro-fibrotic roles in fibrosis, while the TGF-β2 isoform has been described as anti-fibrotic. Decreased production of TGF-β2 is associated with increased expression of a collagen mRNA variant in avian scleroderma, thus its downregulation would lead to a pro-fibrotic state [41].

A small number of studies have addressed the presence of functional activating autoantibodies in SSc, albeit with seemingly contradictory results [1,4-6,19]. Baroni *et al.* found unique and necessary presence of autoantibodies in SSc directed to the PDGFR on fibroblasts [1], while other studies found no difference in PDGFR-binding antibodies between control and SSc groups [5,6] and/or no agonistic activity at all [4,5]. In our cell-based assays, we found variability among the tested samples. Although all SSc and Ct samples immunoprecipitated the PDGFR-β to some degree, certain SSc IgG samples bound the receptor with much greater affinity than the controls. Similarly, the cellular responses to IgG stimulation were more pronounced in SSc IgG-treated cells.

The use of PDGFR kinase inhibitors, however, did not interrupt the signaling activity that we observed suggesting that, although there was PDGFR-binding, this was not the mechanism of action of stimulatory IgG on these cells. Also, it has been reported that SSc autoantibodies can have functional activity on AT1Rs in endothelial cells [19], but we were not able to detect autoantibodies that stimulated signaling through the AT1R in our VSMCs. We did, however, observe signaling that required the catalytic activity of the EGFR. The EGFR is known to have trans-activating activity [42,43] and has also been reported to heterodimerize with other receptors, including the PDGFR [13]. Interestingly, PDGFR and

EGFR are constitutively expressed as heterodimers in primary cultured VSMCs and the activation of early signaling events by PDGF in these cells occurs through the transactivation of the EGFR via a process that is independent of PDGFR kinase activity but dependent on EGFR kinase activity [13]. Heterodimerization of these two receptors has also been shown in a bladder cancer cell line transfected with the PDGFR-β gene [44]. PDGF has also been reported to trans-activate EGFR in skin fibroblasts and in cell-free membranes [45-47]. Indeed, we have also observed PDGFR-EGFR heterodimers in quiescent VSMCs in our lab as previously reported by Saito *et al.*[13] (Supp. Fig. S4). Although SSc-IgG-induced signaling was not blocked with PDGFR kinase inhibitors or an AT1R antagonist, it is conceivable that the EGFR signaling that we observed is one part of a multi-receptor signaling system that may include PDGFR (Fig. 5). In this context, we propose that SSc IgG may engage the PDGFR, a process leading to EGFR activation and subsequent induction of cellular signaling events in a manner independent of the enzymatic activity of the PDGFR, like previously proposed[13]. Our study supports these interesting findings and extends them by showing that this can also occur with activating autoantibodies. How the PDGFR affects the EGFR in the absence of its kinase activity is still unknown but could involve the tyrosine kinase c-Src [14]. In experimental models of SSc, the selective Src kinase inhibitor SU6656 reduced the development of dermal fibrosis [48]. Thus, targeting of Src kinases may be another promising approach in the treatment of SSc.

EGFR-binding autoantibodies have previously been reported in SSc patients, as well as in SLE patients and autoimmune mice [49], but those antibodies had neutralizing or inhibitory effects on A431 cells with the inhibition of DNA synthesis. Taken together with the finding that in VSMCs, a significant proportion of EGFRs and PDGFRs exist as heterodimers [13]

and tests in our lab that show no significant difference in EGFR immunoprecipitation with SSc compared to control IgG (data not shown), we consider unlikely that anti-EGFR autoantibodies are responsible for our observations.

Interestingly, a recent study described SSc IgG that bind to permeabilised VSMCs and induce VSMC contraction [50]. Although the relationship between IgG binding to the VSMC and VSMC reactivity was not discussed, it is known that EGFR activation leads to Ca^{2+} release from intracellular stores [51], and subsequent vascular contraction [52].

The VSMC response to stimulation with purified IgG is consistent with SSc characteristics such as the changes in gene expression reflecting pro-fibrotic changes. TGF-β1 is a pro-fibrotic cytokine and its up-regulation is clearly linked with increased fibrosis [53]. TGF-β2 has been reported to have anti-fibrotic effects in the avian scleroderma model [41], its presence being linked to reduced expression of a pro-collagen mRNA variant. Thus our finding that TGF-β2 is down-regulated by SSc IgG is consistent with a pro-fibrotic state. Also, the increased protein synthesis we observed reflects cell growth and may be relevant in the context of PAH.

We did not detect any relationship between the patient phenotype and stimulatory activity in terms of ERK1/2 or Akt phosphorylation, protein synthesis, gene expression changes, or receptor binding. However, our sample size was relatively small for this type of analysis and we do not feel that any firm conclusions should be drawn until much larger numbers of patients are studied. This would allow better evaluation of the clinical significance of these autoantibodies and of the relationship of these antibodies with the clinical manifestations of SSc.

The presence of increased numbers of autoantibodies define autoimmune diseases and recently autoantibodies have been investigated for functional pathogenic activity. The functional autoantibodies which we describe may have a role in initiating and/or perpetuating the vascular disease of SSc. Further studies are required to better understand the role of the EGFR and to determine if and how the PDGFR and EGFR might interact in response to autoantibody binding. Finally, the rather disappointing results from the use of the non-selective c-Abl, PDGFR, c-kit inhibitor imatinib in SSc clinical trials [[54-56] and reviewed in [57]] may in part be explained by the involvement of EGFR, as we observed in our study. Thus, it may be worthwhile to examine the effects of EGFR inhibitors on the course of the vascular disease of SSc.

2.9 Acknowledgments

We gratefully acknowledge the contributions of the CSRG patients and control individuals involved in this study.

2.9.1 CSRG Recruiting Rheumatologists

M. Baron, McGill University, Montreal, Quebec (lead author; mbaron@rhu.jgh.mcgill.ca); J. Pope, London, University of Western Ontario, London, Ontario; J. Markland, University of Saskatchewan, Saskatoon, Saskatchewan; D. Robinson, University of Manitoba, Winnipeg, Manitoba; N. Jones, University of Edmonton, Edmonton, Alberta; N. Khalidi, McMaster University, Hamilton, Ontario; P. Docherty, The Moncton Hospital, Moncton, New Brunswick; E. Kaminska, McMaster University, Hamilton, Ontario; A. Masetto, Université de Sherbrooke, Sherbrooke, Quebec; E. Sutton, Dalhousie University, Halifax, Nova Scotia; J-P.

Mathieu, Université de Montréal, Montreal, Quebec; M. Hudson, McGill University, Montreal, Quebec; S. Ligier, Université de Montréal, Montreal, Quebec; T. Grodzicky, Université de Montréal, Montreal, Quebec; S. LeClercq, University of Calgary, Calgary, Alberta; C. Thorne, Southlake Regional Health Centre, Newmarket, Ontario; G. Gyger, McGill University, Montreal, Quebec; D. Smith, University of Ottawa, Ottawa, Ontario; and M. Fritzler, Advanced Diagnostics Laboratory and University of Calgary, Calgary, Alberta.

2.10 References

1. Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, et al. (2006) Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. N Engl J Med 354: 2667-2676.

2. Svegliati S, Olivieri A, Campelli N, Luchetti M, Poloni A, et al. (2007) Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. Blood 110: 237-241.

3. Daoussis D, Liossis SN, Yiannopoulos G, Andonopoulos AP (2011) B-cell depletion therapy in systemic sclerosis: experimental rationale and update on clinical evidence. Int J Rheumatol 2011: 214013.

4. Classen JF, Henrohn D, Rorsman F, Lennartsson J, Lauwerys BR, et al. (2009) Lack of evidence of stimulatory autoantibodies to platelet-derived growth factor receptor in patients with systemic sclerosis. Arthritis Rheum 60: 1137-1144.

5. Loizos N, Lariccia L, Weiner J, Griffith H, Boin F, et al. (2009) Lack of detection of agonist activity by antibodies to platelet-derived growth factor receptor alpha in a subset of normal and systemic sclerosis patient sera. Arthritis Rheum 60: 1145-1151.

6. Balada E, Simeon-Aznar CP, Ordi-Ros J, Rosa-Leyva M, Selva-O'Callaghan A, et al. (2008) Anti-PDGFR-alpha antibodies measured by non-bioactivity assays are not specific for systemic sclerosis. Ann Rheum Dis 67: 1027-1029.

7. Kurasawa K, Arai S, Owada T, Maezawa R, Kumano K, et al. (2010) Autoantibodies against platelet-derived growth factor receptor alpha in patients with systemic lupus erythematosus. Mod Rheumatol 20: 458-465.

8. Khimdas S, Harding S, Bonner A, Zummer B, Baron M, et al. (2011) Associations with digital ulcers in a large cohort of systemic sclerosis: results from the Canadian Scleroderma Research Group registry. Arthritis Care Res (Hoboken) 63: 142-149.

9. Lambova S, Muller-Ladner U (2010) Pulmonary arterial hypertension in systemic sclerosis. Autoimmun Rev 9: 761-770.

10. Chan SY, Loscalzo J (2008) Pathogenic mechanisms of pulmonary arterial hypertension. J Mol Cell Cardiol 44: 14-30.

11. Servant MJ, Coulombe P, Turgeon B, Meloche S (2000) Differential regulation of p27(Kip1) expression by mitogenic and hypertrophic factors: Involvement of transcriptional and posttranscriptional mechanisms. J Cell Biol 148: 543-556.

12. Overbeek MJ, Boonstra A, Voskuyl AE, Vonk MC, Vonk-Noordegraaf A, et al. (2011) Platelet-derived growth factor receptor-beta and epidermal growth factor receptor in pulmonary vasculature of systemic sclerosis-associated pulmonary arterial hypertension versus idiopathic pulmonary arterial hypertension and pulmonary veno-occlusive disease: a case-control study. Arthritis Res Ther 13: R61.

13. Saito Y, Haendeler J, Hojo Y, Yamamoto K, Berk BC (2001) Receptor heterodimerization: essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. Mol Cell Biol 21: 6387-6394.

14. Graves LM, Han J, Earp HS, 3rd (2002) Transactivation of the EGF receptor: is the PDGF receptor an unexpected accomplice? Mol Interv 2: 208-212.

15. Douillette A, Bibeau-Poirier A, Gravel SP, Clement JF, Chenard V, et al. (2006) The proinflammatory actions of angiotensin II are dependent on p65 phosphorylation by the IkappaB kinase complex. J Biol Chem 281: 13275-13284.

16. Doyon P, Servant MJ (2010) Tumor necrosis factor receptor-associated factor-6 and ribosomal S6 kinase intracellular pathways link the angiotensin II AT1 receptor to the phosphorylation and activation of the IkappaB kinase complex in vascular smooth muscle cells. J Biol Chem 285: 30708-30718.

17. Duprez DA (2006) Role of the renin-angiotensin-aldosterone system in vascular remodeling and inflammation: a clinical review. J Hypertens 24: 983-991.

18. Saito Y, Berk BC (2001) Transactivation: a novel signaling pathway from angiotensin II to tyrosine kinase receptors. J Mol Cell Cardiol 33: 3-7.

19. Riemekasten G, Philippe A, Nather M, Slowinski T, Muller DN, et al. (2011) Involvement of functional autoantibodies against vascular receptors in systemic sclerosis. Ann Rheum Dis 70: 530-536.

20. Subcommittee (1980) for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma) Arthritis Rheum 23: 581-590.

21. Furst DE, Clements PJ, Steen VD, Medsger TA, Jr., Masi AT, et al. (1998) The modified Rodnan skin score is an accurate reflection of skin biopsy thickness in systemic sclerosis. J Rheumatol 25: 84-88.

22. Medsger TA, Jr., Bombardieri S, Czirjak L, Scorza R, Della Rossa A, et al. (2003) Assessment of disease severity and prognosis. Clin Exp Rheumatol 21: S42-46.

23. Mukerjee D, St George D, Knight C, Davar J, Wells AU, et al. (2004) Echocardiography and pulmonary function as screening tests for pulmonary arterial hypertension in systemic sclerosis. Rheumatology (Oxford) 43: 461-466.

24. Hsu VM, Moreyra AE, Wilson AC, Shinnar M, Shindler DM, et al. (2008) Assessment of pulmonary arterial hypertension in patients with systemic sclerosis: comparison of noninvasive tests with results of right-heart catheterization. J Rheumatol 35: 458-465.

25. Santiago M, Baron M, Hudson M, Burlingame RW, Fritzler MJ (2007) Antibodies to RNA polymerase III in systemic sclerosis detected by ELISA. J Rheumatol 34: 1528-1534.

26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

27. Kuwana M, Okano Y, Pandey JP, Silver RM, Fertig N, et al. (2005) Enzyme-linked immunosorbent assay for detection of anti-RNA polymerase III antibody: analytical accuracy and clinical associations in systemic sclerosis. Arthritis Rheum 52: 2425-2432.

28. Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, et al. (2003) Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. Arterioscler Thromb Vasc Biol 23: 795-801.

29. Dimmeler S, Zeiher AM (2000) Akt takes center stage in angiogenesis signaling. Circ Res 86: 4-5.

30. Kovalenko M, Gazit A, Bohmer A, Rorsman C, Ronnstrand L, et al. (1994) Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. Cancer Res 54: 6106-6114.

31. Tse KF, Novelli E, Civin CI, Bohmer FD, Small D (2001) Inhibition of FLT3-mediated transformation by use of a tyrosine kinase inhibitor. Leukemia 15: 1001-1010.

32. Pardanani A, Tefferi A (2004) Imatinib targets other than bcr/abl and their clinical relevance in myeloid disorders. Blood 104: 1931-1939.

33. Levitzki A, Mishani E (2006) Tyrphostins and other tyrosine kinase inhibitors. Annu Rev Biochem 75: 93-109.

34. Bokemeyer D, Schmitz U, Kramer HJ (2000) Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. Kidney Int 58: 549-558.

35. Verrecchia F, Mauviel A (2007) Transforming growth factor-beta and fibrosis. World J Gastroenterol 13: 3056-3062.

36. Demoulin JB, Essaghir A (2014) PDGF receptor signaling networks in normal and cancer cells. Cytokine Growth Factor Rev.

37. Lurje G, Lenz HJ (2009) EGFR signaling and drug discovery. Oncology 77: 400-410.

38. Shiojima I, Walsh K (2002) Role of Akt signaling in vascular homeostasis and angiogenesis. Circ Res 90: 1243-1250.

39. Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev 12: 502-513.

40. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, et al. (1999) Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem 274: 22699-22704.

41. Prelog M, Scheidegger P, Peter S, Gershwin ME, Wick G, et al. (2005) Diminished transforming growth factor beta2 production leads to increased expression of a profibrotic procollagen alpha2 type I messenger RNA variant in embryonic fibroblasts of UCD-200 chickens, a model for systemic sclerosis. Arthritis Rheum 52: 1804-1811.

42. Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, et al. (1998) Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. J Biol Chem 273: 8890-8896.

43. Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A (2001) Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. Oncogene 20: 1594-1600.

44. Black PC, Brown GA, Dinney CP, Kassouf W, Inamoto T, et al. (2011) Receptor heterodimerization: a new mechanism for platelet-derived growth factor induced resistance to anti-epidermal growth factor receptor therapy for bladder cancer. J Urol 185: 693-700.

45. Walker F, Burgess AW (1991) Reconstitution of the high affinity epidermal growth factor receptor on cell-free membranes after transmodulation by platelet-derived growth factor. J Biol Chem 266: 2746-2752.

46. Decker SJ, Harris P (1989) Effects of platelet-derived growth factor on phosphorylation of the epidermal growth factor receptor in human skin fibroblasts. J Biol Chem 264: 9204-9209.

47. Countaway JL, Girones N, Davis RJ (1989) Reconstitution of epidermal growth factor receptor transmodulation by platelet-derived growth factor in Chinese hamster ovary cells. J Biol Chem 264: 13642-13647.

48. Skhirtladze C, Distler O, Dees C, Akhmetshina A, Busch N, et al. (2008) Src kinases in systemic sclerosis: central roles in fibroblast activation and in skin fibrosis. Arthritis Rheum 58: 1475-1484.

49. Planque S, Zhou YX, Nishiyama Y, Sinha M, O'Connor-Mccourt M, et al. (2003) Autoantibodies to the epidermal growth factor receptor in systemic sclerosis, lupus, and autoimmune mice. FASEB J 17: 136-143.

50. Bussone G, Tamby MC, Calzas C, Kherbeck N, Sahbatou Y, et al. (2012) IgG from patients with pulmonary arterial hypertension and/or systemic sclerosis binds to vascular smooth muscle cells and induces cell contraction. Ann Rheum Dis 71: 596-605.

51. Tinhofer I, Maly K, Dietl P, Hochholdinger F, Mayr S, et al. (1996) Differential Ca2+ signaling induced by activation of the epidermal growth factor and nerve growth factor receptors. J Biol Chem 271: 30505-30509.

52. Matchkov VV, Kudryavtseva O, Aalkjaer C (2012) Intracellular Ca(2)(+) signalling and phenotype of vascular smooth muscle cells. Basic Clin Pharmacol Toxicol 110: 42-48.

53. Border WA, Noble NA (1994) Transforming growth factor beta in tissue fibrosis. N Engl J Med 331: 1286-1292.

54. Mouthon L (2012) SSc in 2011: From mechanisms to medicines. Nat Rev Rheumatol 8: 72-74.

55. Pope J, McBain D, Petrlich L, Watson S, Vanderhoek L, et al. (2011) Imatinib in active diffuse cutaneous systemic sclerosis: Results of a six-month, randomized, double-blind, placebo-controlled, proof-of-concept pilot study at a single center. Arthritis Rheum 63: 3547-3551.

56. Prey S, Ezzedine K, Doussau A, Grandoulier AS, Barcat D, et al. (2012) Imatinib mesylate in scleroderma-associated diffuse skin fibrosis: a phase II multicentre randomized double-blinded controlled trial. Br J Dermatol 167: 1138-1144.

57. Moinzadeh P, Hunzelmann N, Krieg T (2013) Pharmacology and rationale for imatinib in the treatment of scleroderma. Journal of Experimental Pharmacology 2013: 15-22.

2.11 Tables & Figures

Table 1. Characteristics of our SSc cohort (N = 23).

	% or mean ± sd
Mean age, years	48.7 ± 14.5
Female	91%
Disease duration, years	3.5 ± 1.2
Mean Modified Rodnan Skin Score	16.5 ± 11.2
Mean Medsger vascular disease severity	1.9 ± 1.3
Subjects with active vascular ulcers	36%
Subjects with pulmonary hypertension	4.8%
Subjects with anti-centromere antibodies	19%
Subjects with anti-topoisomerase antibodies	29%
Subjects with anti-RNA polymerase III antibodies	43%
Subjects with recent use of immunosuppressive drugs	8.7%

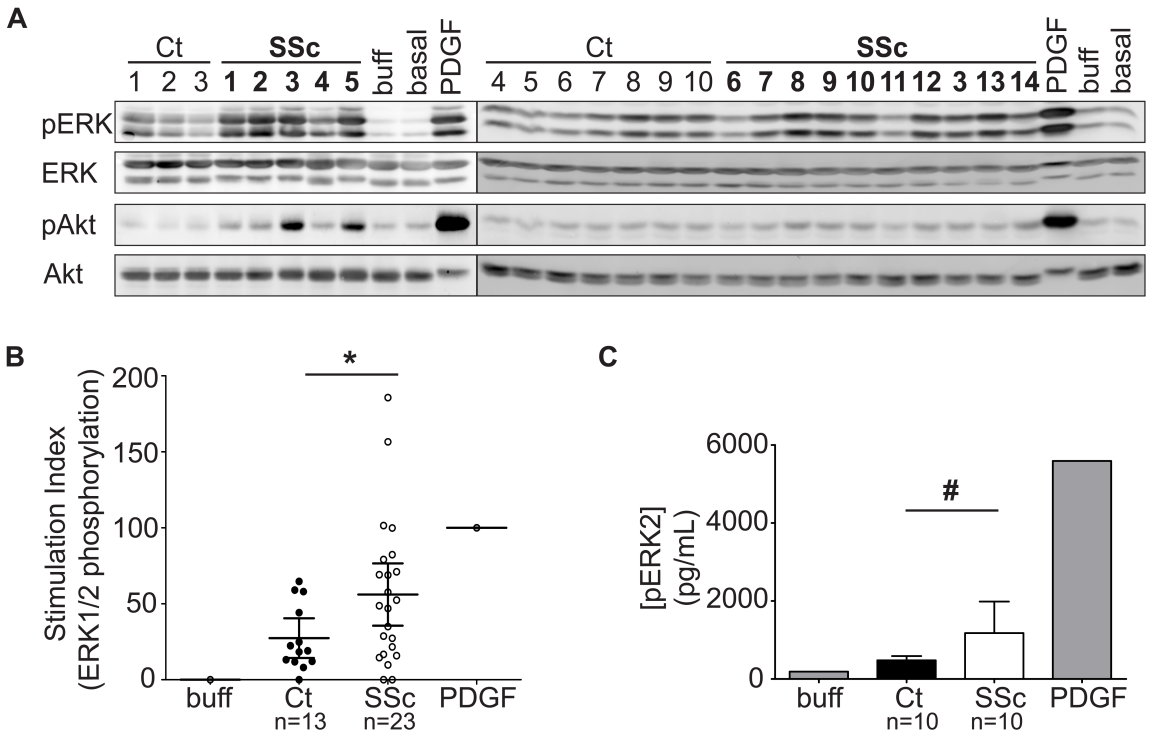

Figure 1.

Effects of purified scleroderma (SSc) and control (Ct) IgG on signaling activity in vascular smooth muscle cells (VSMCs). **A,** Representative Western blots showing ERK1/2 and Akt phosphorylation in quiescent VSMCs treated for 5 minutes with 200 µg/mL purified IgG, the IgG buffer alone, or with 50 ng/mL PDGF. **B,** Densitometric quantification of all immunoblot bands, expressed as a stimulation index scaled to the positive control (PDGF; stimulation index of 100) and negative control (buffer; stimulation index of 0) on the same gel, from 13 control and 23 SSc samples. The stimulation index is calculated as (S-C)/(P-C) x 100 where S, C, and P represent the normalized densitometric pERK1/2 band intensities of a given Sample, the negative Control and the Positive control, respectively. The mean stimulation with 95% confidence intervals is indicated. * p = 0.0474. **C,** ELISA analysis of ERK2 phosphorylation in VSMC stimulated with 200 µg/mL SSc IgG (n=10) or control IgG (n=10). Error bars represent 95% confidence interval. p = 0.0559. buff = buffer; PDGF = platelet-derived growth factor.

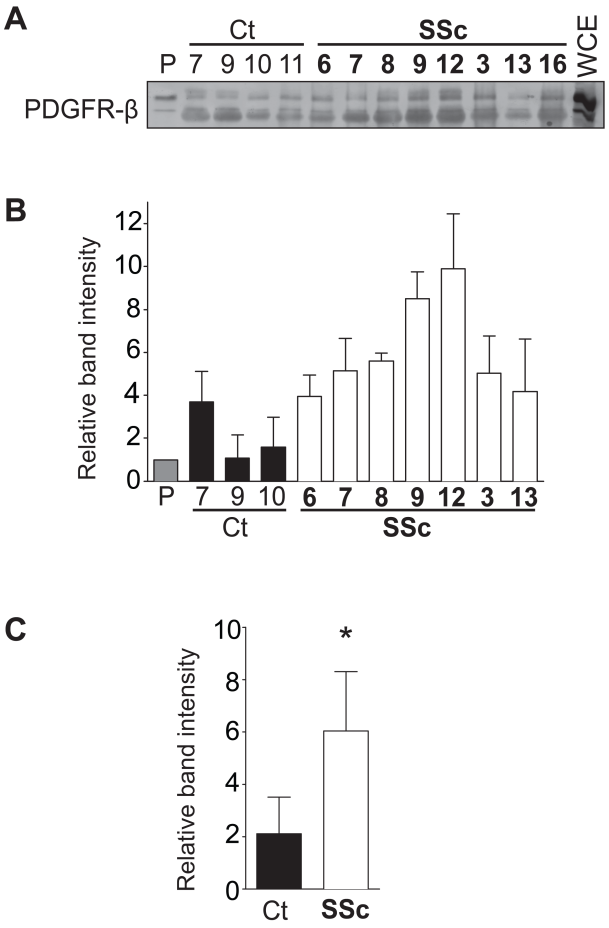

Figure 2.

IgG from systemic sclerosis (SSc) patients bind more to PDGFR than control (Ct) IgG. **A,** Representative immunoblot of PDGFRβ immunoprecipitated with different control or SSc IgG samples, or with a commercially available PDGFRβ antibody (P). The whole cell extract (WCE) was loaded in the far right lane. **B,** Average densitometric analysis of immunoblots from two separate experiments. **C,** Comparison of mean band intensities for control and SSc samples. * $p = 0.0358$.

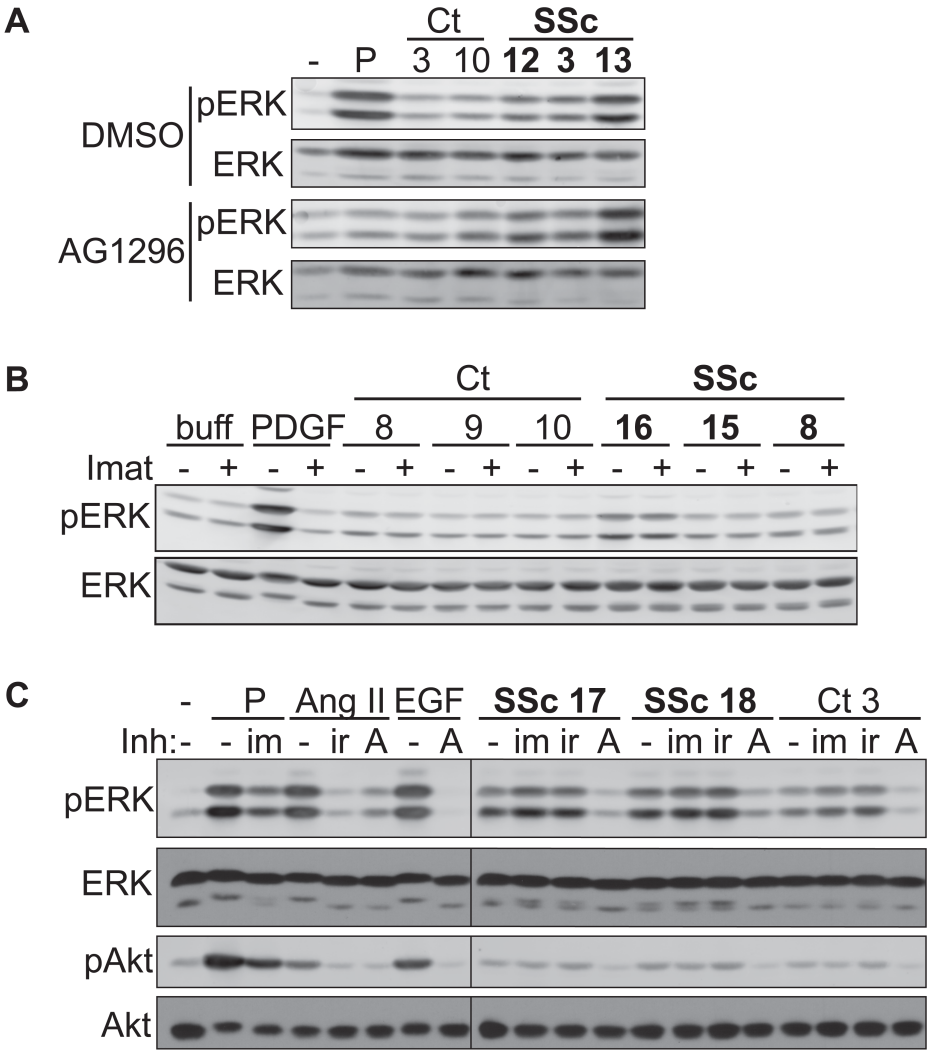

Figure 3.

The phosphotransferase activity of EGFR plays a key role in SSc IgG-induced early signaling events in VSMCs. **A,** Quiescent vascular smooth muscle cells (VSMCs) were pre-treated for 30 minutes with vehicle (0.01% DMSO) or PDGFR-inhibitor AG1296 (5μM) before stimulation with 50 ng/mL PDGF (P) or 200 μg/mL control (Ct) or systemic sclerosis (SSc) patient IgG. **B,** Quiescent VSMCs were pre-treated for 60 minutes with 10μM imatinib mesylate (Imat; +) or with 0.01% DMSO (-), followed by 5-minute stimulation with 50 ng/mL PDGF or 200 μg/mL IgG from control or SSc patients. **C,** Quiescent VSMCs were pre-treated

with inhibitors (Inh; 10μM imatinib (im), 1 μM irbesartan (ir), 250 nM AG1478 (A)), or 0.01% DMSO (-) for 30 minutes prior to stimulation for 5 minutes with 50 ng/mL PDGF (P), 100 nM angiotensin II (AngII), 100 ng/mL epidermal growth factor (EGF), or 200 μg/mL SSc or control IgG. All results shown are representative of at least two experiments with similar results.

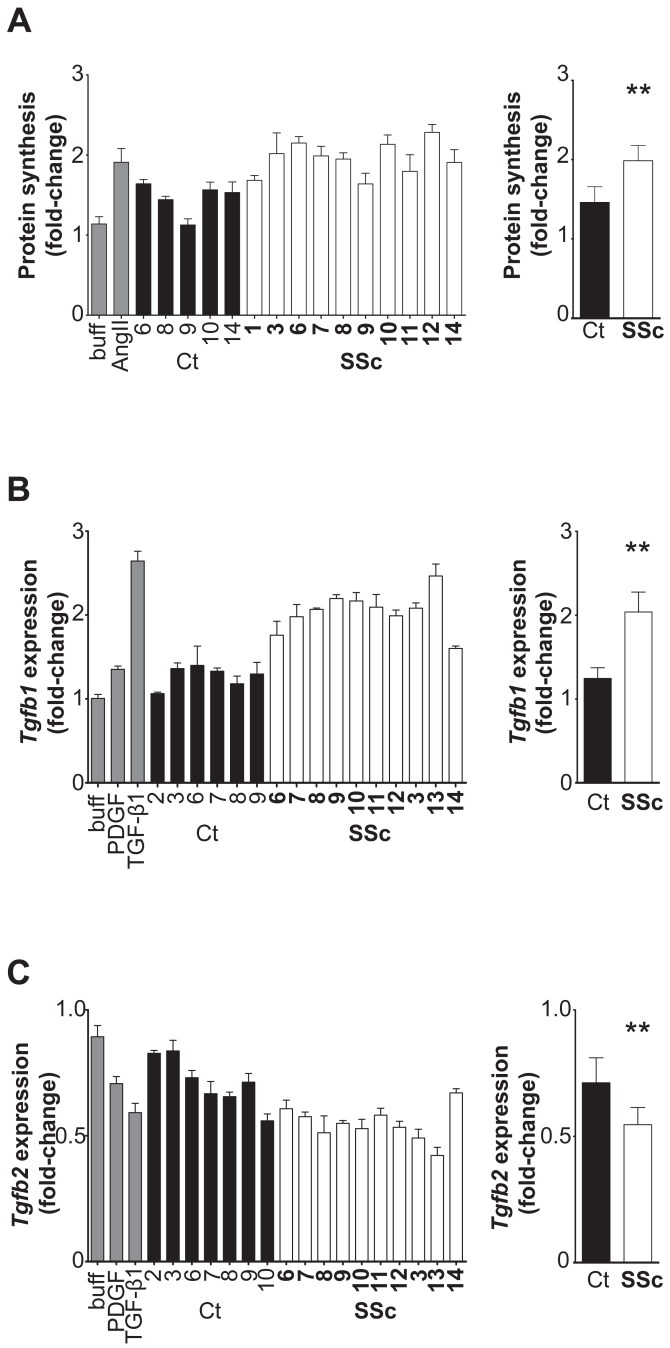

Figure 4.

Systemic sclerosis (SSc) IgG causes growth and profibrotic responses in vascular smooth muscle cells (VSMCs). **A,** Protein synthesis was measured by [^{3}H]-leucine incorporation in quiescent VSMCs stimulated with IgG buffer, angiotensin II (AngII), or 200 µg/mL SSc or

control (Ct) IgG (left), and mean protein synthesis was compared for Ct- and SSc-IgG treated cells (right). Protein synthesis is shown as fold-change with respect to basal. ***p*=0.0008. **B,** Expression of transforming growth factor-β1 mRNA (*Tgfb1*) in VSMCs upon treatment with IgG buffer (buff), 50ng/mL plate-let-derived growth factor (PDGF), 10ng/mL TGF-β1, or 200μg/mL IgG for 2 h (left). Results are normalized to *β-actin* expression and expressed as a fold-change with respect to untreated VSMCs. Mean TGF-β1 expression in Ct-IgG-treated VSMCs compared with that in SSc-IgG treated cells (right). ** $p<0.0001$. **C.** Expression of TGF-β2 mRNA (*Tgfb2*) in VSMCs upon treatment with IgG buffer, 50ng/mL PDGF, 10ng/mL TGF-β1, or IgG for 2 hours (left). Results are normalized to *β-actin* and expressed as a fold-change with respect to untreated VSMCs. Mean TGF-β2 expression in Ct-IgG-treated VSMCs compared with that in SSc-IgG treated cells (right). ** *p*=0.0009. All results shown are representative of at least two experiments. Error bars represent standard deviation.

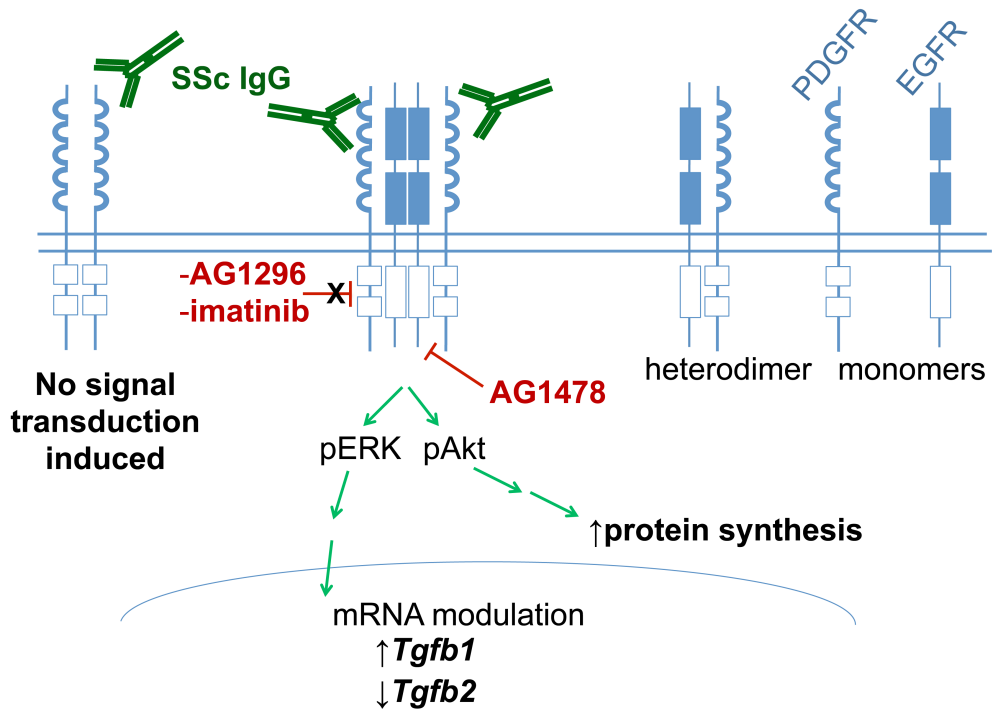

Figure 5.

Platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) are expressed as heterodimers in vascular smooth muscle cells (VSMC) and interestingly, the activation of early signaling events by PDGF in these cells occurs through transactivation of the EGFR via a process that is independent of PDGFR kinase activity but dependent on EGFR kinase activity*. In this context, our data suggest that SSc IgG may bind to the PDGFR allowing transactivation of the EGFR leading to activation of early signaling events, increased protein synthesis, and differential regulation of TGFβ1 and TGFβ2 mRNA expression. The PDGFR inhibiting drugs, AG1296 and imatinib mesylate, do not disrupt SSc-IgG induced ERK1/2 and Akt phosphorylation. EGFR-blocking AG1478 inhibits SSc-IgG induced ERK1/2 and Akt phosphorylation. Image adapted from: *Saito Y, Haendeler J, Hojo Y, Yamamoto K, Berk BC. Receptor heterodimerization: essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. Mol Cell Biol. 2001;21(19):6387-94.

2.11 Supporting Information

Table S1. Details of individual patient samples.

Sample	Sex	Age	ANA	PH	Active vascular ulcers	Peripheral vascular DSS	mRSS	Past use of immuno-suppressives	Stimulation Index[8]
1	F	24	T[1]	n.a.	+	3	22	CS: recent[6]	71
2	F	69	R[2]	+	+	4	43	None	79
3	F	45	R	-	+	2	29	MTX, HCQ: not recent[7]	69
4	F	43	T	-	-	3	6	None	35
5	F	60	R	-	-	1	18	None	69
6	F	46	T	-	+	3	4	None	0
7	F	50	0[3]	-	-	0	11	None	22
8	F	59	n.a.[4]	-	-	0	29	None	27
9	F	69	C[5]	-	-	0	17	None	49
10	F	25	n.a.	-	-	3	30	None	47
11	F	52	C	-	-	0	2	None	0
12	F	50	R	-	-	3	4	None	52
13	F	60	R	-	-	1	18	CS: not recent	82
14	F	75	C	-	-	1	14	None	15
15	F	32	T	-	+	n.a.	n.a.	None	16

16	F	38	0	-	+	n.a.	n.a.	None	29
17	F	33	R	-	-	n.a.	n.a.	None	10
18	F	30	T	-	-	n.a.	n.a.	None	100
19	F	65	C	-	-	2	13	None	186
20	M	53	T	-	+	3	14	MTX: recent	157
21	M	39	R	-	+	3	n.a.	None	58
22	F	60	R	-	n.a.	n.a.	n.a.	None	101
23	F	42	R	n.a.	-	2	30	None	17

ANA, type of anti-nuclear antibodies detected; PH, pulmonary hypertension; DSS, Medsger disease severity score (0=normal, 1=mild, 2=moderate, 3=severe, 4=endstage); mRSS, modified Rodnan skin score; [1]T, anti-topoisomerase I; [2]R, anti-RNA Polymerase III; [3]0, none detected; [4]n.a., data not available; [5]C, anti-centromere; [6]recent = having been stopped less than one year prior to blood draw; [7]"not recent" = having been used at any time more than a year prior to blood draw; CS, corticosteroids; HCQ, hydroxychloroquine; MTX, methotrexate; [8]stimulation index is calculated as (S-C)/(P-C) x 100 where S, C, and P represent the normalized densitometric pERK1/2 band intensities of a given Sample, the negative Control and the Positive control, respectively.

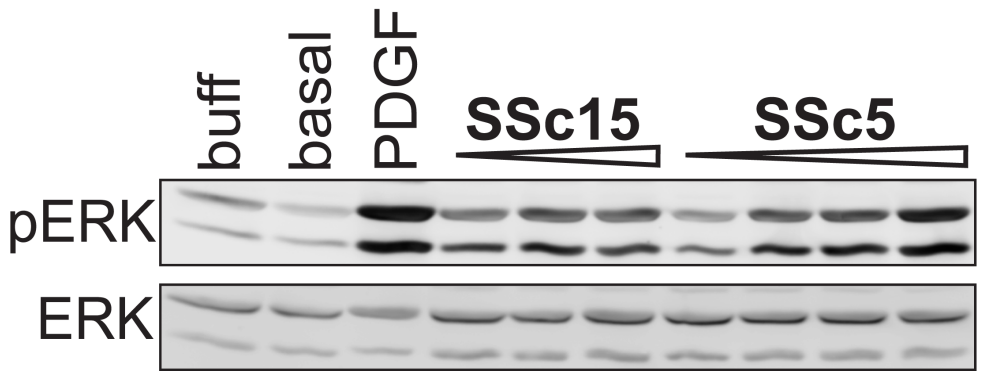

Figure S1.

SSc IgG causes increased ERK phosphorylation in quiescent vascular smooth muscle cells in a dose-dependent manner. Cells were exposed to 50, 100, 150 and in the case of SSc 5 200 μg/mL IgG.

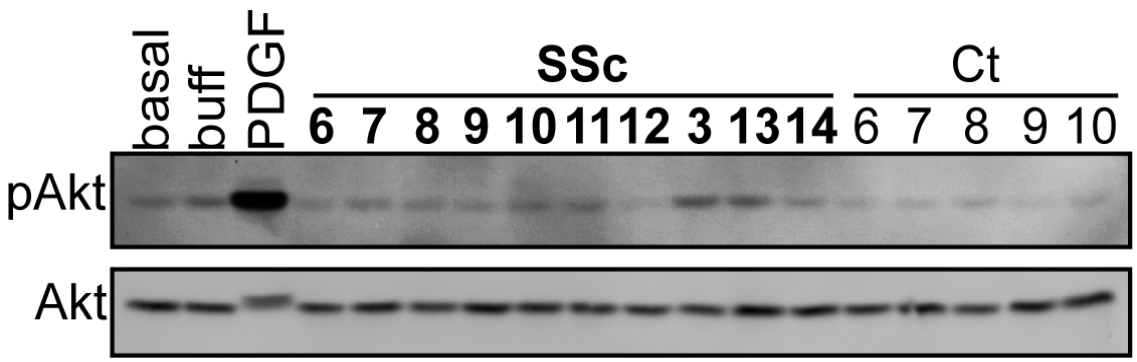

Figure S2.

Effects of purified scleroderma (SSc) and control (Ct) IgG on signaling activity in a primary human fibroblast cell line (MRC5), after 5 minutes of exposure to 200 μg/mL purified IgG, 50 ng/mL PDGF, or the IgG buffer.

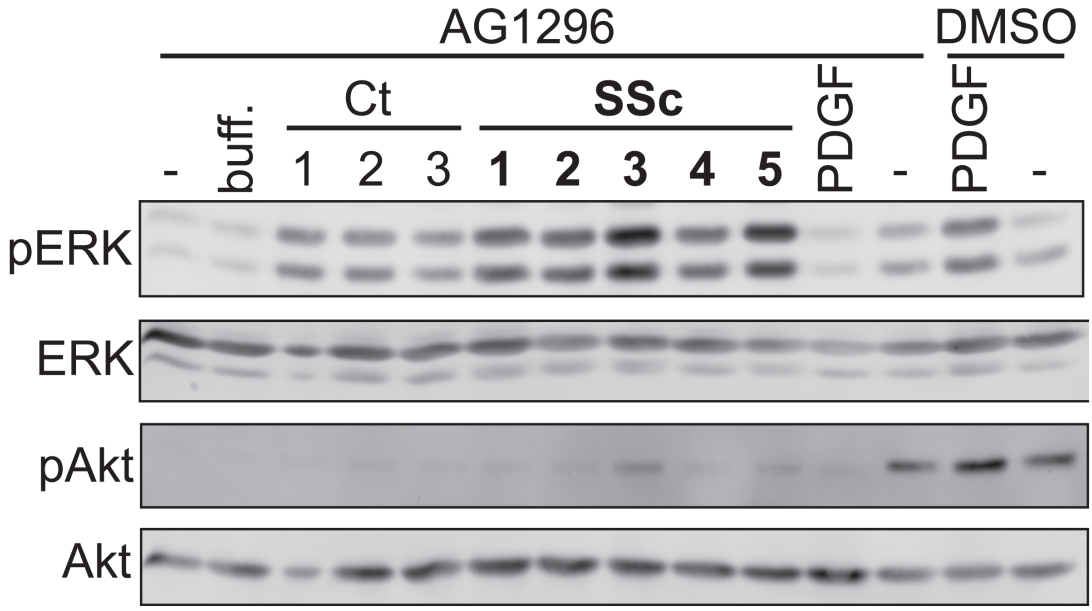

Figure S3.

The platelet-derived growth factor receptor (PDGFR) inhibitor AG1296 does not inhibit the phosphorylation of ERK or Akt in vascular smooth muscle cells (VSMCs) stimulated with IgG from systemic sclerosis (SSc) patients. The inhibitor was effective in completely reducing the signal in response to stimulation with PDGF (compare PDGF/DMSO (lane 13) vs PDGF/AG1296 (lane 11)). Quiescent VSMCs were pre-treated for 30 minutes with vehicle (0.01% DMSO) or PDGFR-inhibitor AG1296 (5μM) before stimulation with 50 ng/mL PDGF (P) or 200 μg/mL control (Ct) or scleroderma (SSc) IgG.

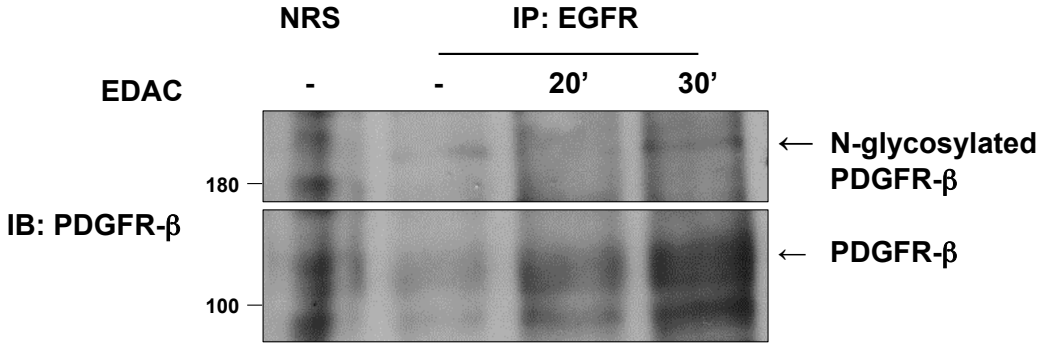

Figure S4.

Constitutive heterodimerization of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) in quiescent vascular smooth muscle cells (VSMCs). Quiescent VSMCs were treated with the cross-linker 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDAC) for 20 or 30 minutes. Cell lysates were prepared as described (Mol Cell Biol. 2001;21(19):6387-94C) and proteins were immunoprecipitated with anti-EGFR antibodies and immunoblotted for PDGFR-β.

Chapter 3. Discussion

3.1. General Discussion

The initial goal of this research project was to determine if the same autoantibodies reported to have agonistic PDGFR activity and subsequent profibrotic effects in fibroblasts[81] might also play a role in the vasculopathic aspect of SSc. We expected to see pathogenic effects on VSMCs since these also express PDGFRs. We observed that stimulatory activity was more prevalent and intense in SSc IgG than in control IgG. However, we were not able to replicate the findings that all SSc IgG activated the PDGFR, and that all normal IgG did not. Similarly, a subset of the IgG fractions isolated from SSc patients clearly immunoprecipitated PDGFRβ, but some SSc samples did not.

The variation among samples that we noted is not surprising. Biologically it is rare to have all-or-nothing contrasts, and this would be even less probable in a population as heterogeneous as SSc patients. Unlike Baroni's initial report, which asserted that all SSc patients, and none of the controls (healthy or having a related condition like lupus) have such autoantibody activity[81], other reports indicate that agonistic anti-PDGFR antibodies reside in healthy subjects[197,198] as well as in SLE patients[196], or were not detectable in their SSc cohort[199]. However, in our study, the SSc IgG activity was significantly greater than that of control IgG in terms of PDGFRβ binding, signal transduction (ERK and Akt phosphorylation), protein synthesis, and TGFβ gene regulation ($p<0.001$). Our results indicated that VSMCs respond to SSc IgG in a manner consistent with SSc pathogenesis, namely that VSMC growth and fibrosis pathways are activated. Interestingly, our findings suggest that the profibrotic state and hypertrophy induced by PDGFR-binding autoantibodies in VSMCs may occur via PDGFR-EGFR heterodimerization and transactivation.

3.1.1. Anti-PDGFR Autoantibodies - Recent Developments

At present the notion of pathogenic anti-PDGFR autoantibodies unique to SSc is still undergoing clarification. The Gabrielli group have since worked on elucidating the epitope(s) targeted by these putative autoantibodies. In particular, they have generated four monoclonal autoantibodies from SSc memory B cells from one SSc patient. These recombinant monoclonal antibodies differed in their effects on PDGFRα. One did not immunoprecipitate the receptor or induce any signaling activity, while the other three immunoprecipitated the receptor, but had different effects (no increase in signaling; activation of Ros, ERK1/2 and Akt; or activation of Ros, ERK1/2, Akt, and Ha-Ras and Type I collagen)[300]. The isolation of these different monoclonal antibodies may facilitate further investigations into the molecular mechanisms underlying autoantibody-stimulated PDGFR activation. However, one must bear in mind the heterogeneous nature of *in-vivo* antibodies. Whatever mechanism of action may be elucidated will not necessarily account for the activity of PDGFR-targeting autoantibodies that recognize different epitopes on the receptor.

The same group has also carried out a study on the effects of SSc IgG on human pulmonary arterial smooth muscle cells, finding increased ROS, migration, proliferation, COL1A1 gene expression, and expression of synthetic phenotype markers (SM-MHC and SM-calponin) mediated by PDGFR[301]. The difference in findings compared to our results may be attributed to the difference in cellular source (*i.e.* we used rat aortic VSMCs) or experimental conditions, such as the duration of

stimulation with IgG[301]. One could add that, as they have shown, a variety of PDGFR-targeting autoantibodies targeting different epitopes and having different levels of PDGFR interactivity are present in SSc IgG. In the small patient populations that constitute most SSc study cohorts, such variations are more conspicuous.

3.1.2. Relevance of Epidermal Growth Factor Receptor Transactivation

Cross-talk between EGFRs and other pathways has been reported extensively[302-304]. Ligand activation of several receptors, such as G protein-coupled receptors endothelin-1 receptor (ET_AR) and angiotensin receptor (AT_1R), and Receptor tyrosine kinases (RTKs), like IGF-1R, can transactivate EGFRs, albeit indirectly through the stimulation of metalloproteinases which liberate membrane-bound EGFR ligands or through activation of EGFR's tyrosine kinase domain by the non-receptor tyrosine kinase Src[305-311]. Src is also known to mediate TNF-α receptor transactivation of EGFR[312]

There are a few studies that support interactions between PDGFRβ and EGFRs. PDGF was shown to stimulate EGFR serine phosphorylation, purportedly regulating EGF-EGFR affinity[313-315]. PDGFRβ and EGFR have both been isolated from caveola-containing membranes[316,317], meaning they are likely localized in proximity to each other. When transfected into Cos-7 cells, in the absence of any ligand, EGFR and PDGFRβ coprecipitate, and upon treatment with EGF, PDGFRβ has increased phosphorylation[318]. EGFR has also been reported to be transactivated following PDGF stimulation[313,314,319-321]. In murine B82L fibroblasts transfected with wild-type EGFR, PDGF stimulated tyrosine phosphorylation in EGFR, and this activation was required for increased cellular migration[321]. Similarly, the enzymatic activity of EGFR was required for p21-activated kinase (PAK) activity in response

to PDGF stimulation[322]. And Saito *et al*. demonstrated that in cells that express both PDGFRβ and EGFR, these receptors associate into heterodimers even under basal conditions. Interestingly, they report that antioxidants and Src inhibitors abolish EGFR- PDGFRβ heterodimer formation and signaling, suggesting ROS has some role in stabilizing these receptor complexes. Ligand-independent EGFR- PDGFRβ heterodimerization was also observed in EGFR-expressing bladder cancer cells transfected with PDGFRβ[267]. Despite the evidence already discovered, the notion of PDGFR-EGFR heterodimerization has not received a great deal of attention.

In our study, SSc patient IgG caused profibrotic changes and growth in VSMCs, and although SSc IgG bound to the PDGFRβ, PDGFR-targeting tyrosine kinase inhibitors did not abrogate the induced phenotype. However, EGFR-specific tyrosine-kinase inhibitor AG1478 did block the activity of SSc IgG in VSMCs. Based on previous findings of PDGFR-EGFR interaction and transactivation, we suggest as a possible explanation the model in Figure 5, in which SSc IgG directly binds to the PDGFR, which then transactivates EGFR, its heterodimeric partner.

To provide support to this model, it would be useful to co-immunoprecipitate the heterodimerized receptors. In our immunoprecipitation of VSMC lysates using SSc IgG, we detected PDGFR but not EGFR. It is likely that the conditions involved in immunoprecipitating these proteins may destabilize the PDGFR-EGFR heterodimers, thus it would be necessary to use a cross-linking agent to stabilize the interaction.

Also, it may be interesting to determine which EGFR subtype(s) might be involved in the response to SSc IgG. Since the kinase activity of EGFR was necessary for SSc-IgG induced signaling, the kinase-inactive ErbB3 is not likely to be involved. It would be

speculation, but one might consider that ErbB2, without any known cognate ligands, may have a biological role in heterodimerization with other receptors. Notably, we have not excluded the involvement of other non-receptor proteins.

Previous studies have tended to focus on PDGFR-α. Indeed, the research group most vested in deepening our understanding of anti-PDGFR autoantibodies has developed a PDGFR-α expressing fibroblast cell line (Fα), which they use extensively in their research[81,300]. For example, they have used PDGFR-α fragments derived from these cells in the development of monoclonal anti-PDGFR-α derived from one SSc patient's serum[300]. However, PDGFR-β has not been excluded from interacting with SSc IgG, and given the redundancy and overlap of PDGFR subtype activities, and the fact that PDGFR-β is important in vascular development and integrity[323,324] we focused on PDGFR-β in our study. In the PDGFR-EGFR heterodimerization-transactivation paper by Saito *et al.* [268], only PDGFR-β was assessed. It may be useful to determine if there are any differences between the α and β receptor subtypes in the context of SSc IgG responses and EGFR-PDGFR heterodimerizations.

3.2. Conclusion

Although much has been learned about SSc, many elements about its pathogenesis are still not well understood. In the research of the PDGFR-stimulating autoantibodies hypothesis, there is still much work to be done. Researchers are trying to better understand the profibrotic activity of these autantibodies, using monoclonal antibodies derived from SSc patient sera. This will be helpful in understanding the cellular and molecular mechanisms taking place. In reality, though, patients have multiple autoantibodies in their circulation which target a mixture of

epitopes. The true *in-vivo* situation will be affected by many factors. Similarly, the use of receptor-inhibiting molecules, developed and brought to market as treatments for less rare diseases, like cancer, is an attractive approach. PDGFR-targeting inhibitors are being tested, however given the PDGFR-EGFR interaction that we observed, it may be worthwhile to consider the feasibility of testing EGFR-inhibitors. Similarly, the PDGFR-EGFR heterodimeric interaction that we observed may merit further research as it may offer novel drug targets that may be relevant for patients affected with SSc, or other diseases with vasculopathic features.

REFERENCES

1. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest* 2007; 117: 557-567.
2. Bernatsky S, Joseph L, Pineau CA, Belisle P, Hudson M, Clarke AE. Scleroderma prevalence: demographic variations in a population-based sample. *Arthritis Rheum* 2009; 61: 400-404.
3. Andersen L, Davis M. Prevalence of Skin and Skin-Related Diseases in the Rochester Epidemiology Project and a Comparison with Other Published Prevalence Studies. *Dermatology* 2016; 232: 344-352.
4. Silman A. Scleroderma--demographics and survival. *J Rheumatol Suppl* 1997 May; 48: 58-61.
5. Mayes MD, Lacey JV, Jr., Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, Schottenfeld D. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. *Arthritis Rheum* 2003; 48: 2246-2255.
6. Thompson A, Pope J. Increased prevalence of scleroderma in southwestern Ontario: a cluster analysis. *J Rheumatol* 2002; 29: 1867-1873.
7. Chifflot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and prevalence of systemic sclerosis: a systematic literature review. *Semin Arthritis Rheum* 2008; 37: 223-235.
8. Panopoulos ST, Bournia VK, Sfikakis PP. Is vasculopathy associated with systemic sclerosis more severe in men? *J Rheumatol* 2013; 40: 46-51.
9. Steen VD, Medsger TA. Changes in causes of death in systemic sclerosis, 1972-2002. *Ann Rheum Dis* 2007; 66: 940-944.
10. Ferri C, Sebastiani M, Lo Monaco A, Iudici M, Giuggioli D, Furini F, Manfredi A, Cuomo G, Spinella A, Colaci M, Govoni M, Valentini G. Systemic sclerosis evolution of disease pathomorphosis and survival. Our experience on Italian patients' population and review of the literature. *Autoimmun Rev* 2014; 13: 1026-1034.
11. Steen VD, Costantino JP, Shapiro AP, Medsger TA, Jr. Outcome of renal crisis in systemic sclerosis: relation to availability of angiotensin converting enzyme (ACE) inhibitors. *Ann Intern Med* 1990; 113: 352-357.
12. Nikpour M, Baron M. Mortality in systemic sclerosis: lessons learned from population-based and observational cohort studies. *Curr Opin Rheumatol* 2014; 26: 131-137.
13. Desbois AC, Cacoub P. Systemic sclerosis: An update in 2016. *Autoimmun Rev* 2016; 15: 417-426.
14. Rodnan GP, Myerowitz RL, Justh GO. Morphologic changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynaud phenomenon. *Medicine (Baltimore)* 1980; 59: 393-408.
15. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Rheumatology (Oxford)* 2005; 44: 587-596.
16. van Lankveld WG, Vonk MC, Teunissen H, van den Hoogen FH. Appearance self-esteem in systemic sclerosis--subjective experience of skin deformity and its relationship with physician-assessed skin involvement, disease status and psychological variables. *Rheumatology (Oxford)* 2007; 46: 872-876.

17. Alantar A, Cabane J, Hachulla E, Princ G, Ginisty D, Hassin M, Sorel M, Maman L, Pilat A, Mouthon L. Recommendations for the care of oral involvement in patients with systemic sclerosis. *Arthritis Care Res (Hoboken)* 2011; 63: 1126-1133.
18. Galluccio F, Matucci-Cerinic M. Two faces of the same coin: Raynaud phenomenon and digital ulcers in systemic sclerosis. *Autoimmun Rev* 2011; 10: 241-243.
19. Daoussis D, Antonopoulos I, Liossis SN, Yiannopoulos G, Andonopoulos AP. Treatment of systemic sclerosis-associated calcinosis: a case report of rituximab-induced regression of CREST-related calcinosis and review of the literature. *Semin Arthritis Rheum* 2012; 41: 822-829.
20. Khanna D, Denton CP. Evidence-based management of rapidly progressing systemic sclerosis. *Best Pract Res Clin Rheumatol* 2010; 24: 387-400.
21. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., Rowell N, Wollheim F. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; 15: 202-205.
22. LeRoy EC, Medsger TA, Jr. Criteria for the classification of early systemic sclerosis. *J Rheumatol* 2001; 28: 1573-1576.
23. Steen VD, Medsger TA, Jr. Severe organ involvement in systemic sclerosis with diffuse scleroderma. *Arthritis Rheum* 2000; 43: 2437-2444.
24. Varga J, Hinchcliff M. Connective tissue diseases: systemic sclerosis: beyond limited and diffuse subsets? *Nat Rev Rheumatol* 2014; 10: 200-202.
25. Moinzadeh P, Aberer E, Ahmadi-Simab K, Blank N, Distler JH, Fierlbeck G, Genth E, Guenther C, Hein R, Henes J, Herich L, Herrgott I, Koetter I, Kreuter A, Krieg T, Kuhr K, Lorenz HM, Meier F, Melchers I, Mensing H, Mueller-Ladner U, Pfeiffer C, Riemekasten G, Sardy M, Schmalzing M, Sunderkoetter C, Susok L, Tarner IH, Vaith P, Worm M, Wozel G, Zeidler G, Hunzelmann N, all participating Dc. Disease progression in systemic sclerosis-overlap syndrome is significantly different from limited and diffuse cutaneous systemic sclerosis. *Ann Rheum Dis* 2015; 74: 730-737.
26. Milano A, Pendergrass SA, Sargent JL, George LK, McCalmont TH, Connolly MK, Whitfield ML. Molecular subsets in the gene expression signatures of scleroderma skin. *PLoS One* 2008; 3: e2696.
27. Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K. The prevalence and clinical significance of anti-U1 RNA antibodies in patients with systemic sclerosis. *J Invest Dermatol* 2003; 120: 204-210.
28. Greidinger EL, Casciola-Rosen L, Morris SM, Hoffman RW, Rosen A. Autoantibody recognition of distinctly modified forms of the U1-70-kd antigen is associated with different clinical disease manifestations. *Arthritis Rheum* 2000; 43: 881-888.
29. Johnson SR. New ACR EULAR guidelines for systemic sclerosis classification. *Curr Rheumatol Rep* 2015; 17: 32.
30. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). . *Arthritis Rheum* 1980; 23: 581-590.
31. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, Matucci-Cerinic M, Naden RP, Medsger TA, Jr., Carreira PE, Riemekasten G, Clements PJ, Denton CP, Distler O, Allanore Y, Furst DE, Gabrielli A, Mayes MD, van Laar JM, Seibold JR, Czirjak L, Steen VD, Inanc M, Kowal-Bielecka O, Muller-Ladner U, Valentini G, Veale DJ, Vonk MC, Walker UA, Chung L, Collier DH, Csuka ME,

Fessler BJ, Guiducci S, Herrick A, Hsu VM, Jimenez S, Kahaleh B, Merkel PA, Sierakowski S, Silver RM, Simms RW, Varga J, Pope JE. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum* 2013; 65: 2737-2747.
32. Varga J. Systemic Sclerosis (Scleroderma) and Related Disorders. In: Kasper D, Fauci A, Hauser S, Longo D, Jameson JL, Loscalzo J, editors. Harrison's Principles of Internal Medicine, 19e. New York, NY: McGraw-Hill Education; 2015.
33. Mayes M. Brief Overview of Scleroderma: Localized Scleroderma and Systemic Sclerosis (SSc). In: Mayes M, editor. A Visual Guide to Scleroderma and Approach to Treatment: Springer-Verlag New York; 2014.
34. Diab S, Dostrovsky N, Hudson M, Tatibouet S, Fritzler MJ, Baron M, Khalidi N, Canadian Scleroderma Research G. Systemic sclerosis sine scleroderma: a multicenter study of 1417 subjects. *J Rheumatol* 2014; 41: 2179-2185.
35. Poormoghim H, Lucas M, Fertig N, Medsger TA, Jr. Systemic sclerosis sine scleroderma: demographic, clinical, and serologic features and survival in forty-eight patients. *Arthritis Rheum* 2000; 43: 444-451.
36. Chung L, Utz PJ. Antibodies in scleroderma: direct pathogenicity and phenotypic associations. *Curr Rheumatol Rep* 2004; 6: 156-163.
37. Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, Mayes MD. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. *Arthritis Rheum* 2001; 44: 1359-1362.
38. McNearney TA, Reveille JD, Fischbach M, Friedman AW, Lisse JR, Goel N, Tan FK, Zhou X, Ahn C, Feghali-Bostwick CA, Fritzler M, Arnett FC, Mayes MD. Pulmonary involvement in systemic sclerosis: associations with genetic, serologic, sociodemographic, and behavioral factors. *Arthritis Rheum* 2007; 57: 318-326.
39. Arnett FC, Howard RF, Tan F, Moulds JM, Bias WB, Durban E, Cameron HD, Paxton G, Hodge TJ, Weathers PE, Reveille JD. Increased prevalence of systemic sclerosis in a Native American tribe in Oklahoma. Association with an Amerindian HLA haplotype. *Arthritis Rheum* 1996; 39: 1362-1370.
40. Zhou X, Lee JE, Arnett FC, Xiong M, Park MY, Yoo YK, Shin ES, Reveille JD, Mayes MD, Kim JH, Song R, Choi JY, Park JA, Lee YJ, Lee EY, Song YW, Lee EB. HLA-DPB1 and DPB2 are genetic loci for systemic sclerosis: a genome-wide association study in Koreans with replication in North Americans. *Arthritis Rheum* 2009; 60: 3807-3814.
41. Dieude P, Guedj M, Wipff J, Avouac J, Fajardy I, Diot E, Granel B, Sibilia J, Cabane J, Mouthon L, Cracowski JL, Carpentier PH, Hachulla E, Meyer O, Kahan A, Boileau C, Allanore Y. Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: a new perspective for pulmonary fibrosis. *Arthritis Rheum* 2009; 60: 225-233.
42. Dieude P, Dawidowicz K, Guedj M, Legrain Y, Wipff J, Hachulla E, Diot E, Sibilia J, Mouthon L, Cabane J, Amoura Z, Crakowski JL, Carpentier P, Avouac J, Meyer O, Kahan A, Boileau C, Allanore Y. Phenotype-haplotype correlation of IRF5 in systemic sclerosis: role of 2 haplotypes in disease severity. *J Rheumatol* 2010; 37: 987-992.
43. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Hikami K, Kawamoto M, Fujimoto M, Takehara K, Sato S, Hara M, Tsuchiya N. Association of a functional

polymorphism in the IRF5 region with systemic sclerosis in a Japanese population. *Arthritis Rheum* 2009; 60: 1845-1850.
44. Kawaguchi Y, Tochimoto A, Hara M, Kawamoto M, Sugiura T, Saito S, Kamatani N. Contribution of single nucleotide polymorphisms of the IL1A gene to the cleavage of precursor IL-1alpha and its transcription activity. *Immunogenetics* 2007; 59: 441-448.
45. Buyuk U, Ates O, Dalyan L, Musellim B, Ongen G, Topal-Sarikaya A. Analysis of transforming growth factor beta 1 (TGF-beta1) gene polymorphisms in Turkish patients with scleroderma. *Cell Biochem Funct* 2010; 28: 274-277.
46. Tan FK, Wang N, Kuwana M, Chakraborty R, Bona CA, Milewicz DM, Arnett FC. Association of fibrillin 1 single-nucleotide polymorphism haplotypes with systemic sclerosis in Choctaw and Japanese populations. *Arthritis Rheum* 2001; 44: 893-901.
47. Otieno FG, Lopez AM, Jimenez SA, Gentiletti J, Artlett CM. Allograft inflammatory factor-1 and tumor necrosis factor single nucleotide polymorphisms in systemic sclerosis. *Tissue Antigens* 2007; 69: 583-591.
48. Morita H, Hayashi D, Nagai R. CTGF polymorphism associated with systemic sclerosis. *N Engl J Med* 2008; 358: 308; author reply 309.
49. Diot E, Lesire V, Guilmot JL, Metzger MD, Pilore R, Rogier S, Stadler M, Diot P, Lemarie E, Lasfargues G. Systemic sclerosis and occupational risk factors: a case-control study. *Occup Environ Med* 2002; 59: 545-549.
50. Hamamdzic D, Kasman LM, LeRoy EC. The role of infectious agents in the pathogenesis of systemic sclerosis. *Curr Opin Rheumatol* 2002; 14: 694-698.
51. Ferri C, Longombardo G, Azzi A, Zakrzewska K. Parvovirus B19 and systemic sclerosis. *Clin Exp Rheumatol* 1999; 17: 267-268.
52. Pandey JP. Immunoglobulin GM genes and IgG antibodies to cytomegalovirus in patients with systemic sclerosis. *Clin Exp Rheumatol* 2004; 22: S35-37.
53. Yazawa N, Fujimoto M, Kikuchi K, Kubo M, Ihn H, Sato S, Tamaki T, Tamaki K. High seroprevalence of Helicobacter pylori infection in patients with systemic sclerosis: association with esophageal involvement. *J Rheumatol* 1998; 25: 650-653.
54. Adams Waldorf KM, Nelson JL. Autoimmune disease during pregnancy and the microchimerism legacy of pregnancy. *Immunol Invest* 2008; 37: 631-644.
55. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 1996; 93: 705-708.
56. Lo YM, Lo ES, Watson N, Noakes L, Sargent IL, Thilaganathan B, Wainscoat JS. Two-way cell traffic between mother and fetus: biologic and clinical implications. *Blood* 1996; 88: 4390-4395.
57. Maloney S, Smith A, Furst DE, Myerson D, Rupert K, Evans PC, Nelson JL. Microchimerism of maternal origin persists into adult life. *J Clin Invest* 1999; 104: 41-47.
58. Lambert NC, Lo YM, Erickson TD, Tylee TS, Guthrie KA, Furst DE, Nelson JL. Male microchimerism in healthy women and women with scleroderma: cells or circulating DNA? A quantitative answer. *Blood* 2002; 100: 2845-2851.
59. Nelson JL, Furst DE, Maloney S, Gooley T, Evans PC, Smith A, Bean MA, Ober C, Bianchi DW. Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. *Lancet* 1998; 351: 559-562.

60. Barnes JK, Mayes MD. Epidemiology and Environmental Risk Factors. In: Varga J, Denton CP, Wigley FM, editors. Scleroderma: From Pathogenesis to Comprehensive Management. Boston, MA: Springer US; 2012. p. 17-28.
61. Alba MA, Velasco C, Simeon CP, Fonollosa V, Trapiella L, Egurbide MV, Saez L, Castillo MJ, Callejas JL, Camps MT, Tolosa C, Rios JJ, Freire M, Vargas JA, Espinosa G, Registry R. Early- versus late-onset systemic sclerosis: differences in clinical presentation and outcome in 1037 patients. *Medicine (Baltimore)* 2014; 93: 73-81.
62. Denton CP. Therapeutic targets in systemic sclerosis. *Arthritis Res Ther* 2007; 9 Suppl 2: S6.
63. Abraham DJ, Varga J. Scleroderma: from cell and molecular mechanisms to disease models. *Trends in immunology* 2005; 26: 587-595.
64. Enzerink A, Salmenpera P, Kankuri E, Vaheri A. Clustering of fibroblasts induces proinflammatory chemokine secretion promoting leukocyte migration. *Mol Immunol* 2009; 46: 1787-1795.
65. Chizzolini C, Raschi E, Rezzonico R, Testoni C, Mallone R, Gabrielli A, Facchini A, Del Papa N, Borghi MO, Dayer JM, Meroni PL. Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis. *Arthritis Rheum* 2002; 46: 1602-1613.
66. Abraham D, Distler O. How does endothelial cell injury start? The role of endothelin in systemic sclerosis. *Arthritis Res Ther* 2007; 9 Suppl 2: S2.
67. Fleischmajer R, Perlish JS, Shaw KV, Pirozzi DJ. Skin capillary changes in early systemic scleroderma. Electron microscopy and "in vitro" autoradiography with tritiated thymidine. *Arch Dermatol* 1976; 112: 1553-1557.
68. Abraham DJ, Krieg T, Distler J, Distler O. Overview of pathogenesis of systemic sclerosis. *Rheumatology (Oxford)* 2009; 48 Suppl 3: iii3-7.
69. Kahaleh MB, Sherer GK, LeRoy EC. Endothelial injury in scleroderma. *J Exp Med* 1979; 149: 1326-1335.
70. Blann AD, Illingworth K, Jayson MI. Mechanisms of endothelial cell damage in systemic sclerosis and Raynaud's phenomenon. *J Rheumatol* 1993; 20: 1325-1330.
71. Carpentier PH, Maricq HR. Microvasculature in systemic sclerosis. *Rheum Dis Clin North Am* 1990; 16: 75-91.
72. Cutolo M, Sulli A, Secchi ME, Paolino S, Pizzorni C. Nailfold capillaroscopy is useful for the diagnosis and follow-up of autoimmune rheumatic diseases. A future tool for the analysis of microvascular heart involvement? *Rheumatology (Oxford)* 2006; 45 Suppl 4: iv43-46.
73. Fleischmajer R, Perlish JS, Duncan M. Scleroderma. A model for fibrosis. *Arch Dermatol* 1983; 119: 957-962.
74. Grassi W, Core P, Carlino G, Blasetti P, Cervini M. Labial capillary microscopy in systemic sclerosis. *Ann Rheum Dis* 1993; 52: 564-569.
75. Fleischmajer R, Perlish JS. Capillary alterations in scleroderma. *J Am Acad Dermatol* 1980; 2: 161-170.
76. Kahaleh MB. Vascular involvement in systemic sclerosis (SSc). *Clin Exp Rheumatol* 2004; 22: S19-23.
77. LeRoy EC. Systemic sclerosis. A vascular perspective. *Rheum Dis Clin North Am* 1996; 22: 675-694.

78. Rzucidlo EM, Martin KA, Powell RJ. Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg* 2007; 45 Suppl A: A25-32.
79. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; 39: 44-84.
80. Riccieri V, Spadaro A, Fuksa L, Firuzi O, Saso L, Valesini G. Specific oxidative stress parameters differently correlate with nailfold capillaroscopy changes and organ involvement in systemic sclerosis. *Clin Rheumatol* 2008; 27: 225-230.
81. Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, Fraticelli P, Sambo P, Funaro A, Kazlauskas A, Avvedimento EV, Gabrielli A. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med* 2006; 354: 2667-2676.
82. Negi VS, Tripathy NK, Misra R, Nityanand S. Antiendothelial cell antibodies in scleroderma correlate with severe digital ischemia and pulmonary arterial hypertension. *J Rheumatol* 1998; 25: 462-466.
83. Hill MB, Phipps JL, Cartwright RJ, Milford Ward A, Greaves M, Hughes P. Antibodies to membranes of endothelial cells and fibroblasts in scleroderma. *Clinical and experimental immunology* 1996; 106: 491-497.
84. Pignone A, Scaletti C, Matucci-Cerinic M, Vazquez-Abad D, Meroni PL, Del Papa N, Falcini F, Generini S, Rothfield N, Cagnoni M. Anti-endothelial cell antibodies in systemic sclerosis: significant association with vascular involvement and alveolo-capillary impairment. *Clin Exp Rheumatol* 1998; 16: 527-532.
85. Youinou P, Revelen R, Bordron A. Is antiendothelial cell antibody the murder weapon in systemic sclerosis? *Clin Exp Rheumatol* 1999; 17: 35-36.
86. Bordron A, Dueymes M, Levy Y, Jamin C, Leroy JP, Piette JC, Shoenfeld Y, Youinou PY. The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. *J Clin Invest* 1998; 101: 2029-2035.
87. Arends SJ, Damoiseaux JG, Duijvestijn AM, Debrus-Palmans L, Boomars KA, Brunner-La Rocca HP, Cohen Tervaert JW, van Paassen P. Functional implications of IgG anti-endothelial cell antibodies in pulmonary arterial hypertension. *Autoimmunity* 2013; 46: 463-470.
88. Murdaca G, Colombo BM, Cagnati P, Gulli R, Spano F, Puppo F. Endothelial dysfunction in rheumatic autoimmune diseases. *Atherosclerosis* 2012; 224: 309-317.
89. Morgan-Rowe L, Nikitorowicz J, Shiwen X, Leask A, Tsui J, Abraham D, Stratton R. Thrombospondin 1 in hypoxia-conditioned media blocks the growth of human microvascular endothelial cells and is increased in systemic sclerosis tissues. *Fibrogenesis Tissue Repair* 2011; 4: 13.
90. Frommer KW, Muller-Ladner U. Expression and function of ETA and ETB receptors in SSc. *Rheumatology (Oxford)* 2008; 47 Suppl 5: v27-28.
91. Morelli S, Ferri C, Di Francesco L, Baldoncini R, Carlesimo M, Bottoni U, Properzi G, Santucci A. Plasma endothelin-1 levels in patients with systemic sclerosis: influence of pulmonary or systemic arterial hypertension. *Ann Rheum Dis* 1995; 54: 730-734.
92. Cambrey AD, Harrison NK, Dawes KE, Southcott AM, Black CM, du Bois RM, Laurent GJ, McAnulty RJ. Increased levels of endothelin-1 in bronchoalveolar lavage fluid from patients with systemic sclerosis contribute to fibroblast mitogenic activity in vitro. *Am J Respir Cell Mol Biol* 1994; 11: 439-445.

93. Kawaguchi Y, Suzuki K, Hara M, Hidaka T, Ishizuka T, Kawagoe M, Nakamura H. Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis. *Ann Rheum Dis* 1994; 53: 506-510.
94. Vancheeswaran R, Azam A, Black C, Dashwood MR. Localization of endothelin-1 and its binding sites in scleroderma skin. *J Rheumatol* 1994; 21: 1268-1276.
95. Vancheeswaran R, Magoulas T, Efrat G, Wheeler-Jones C, Olsen I, Penny R, Black CM. Circulating endothelin-1 levels in systemic sclerosis subsets--a marker of fibrosis or vascular dysfunction? *J Rheumatol* 1994; 21: 1838-1844.
96. Morelli S, Ferri C, Polettini E, Bellini C, Gualdi GF, Pittoni V, Valesini G, Santucci A. Plasma endothelin-1 levels, pulmonary hypertension, and lung fibrosis in patients with systemic sclerosis. *Am J Med* 1995; 99: 255-260.
97. Richard V, Solans V, Favre J, Henry JP, Lallemand F, Thuillez C, Marie I. Role of endogenous endothelin in endothelial dysfunction in murine model of systemic sclerosis: tight skin mice 1. *Fundam Clin Pharmacol* 2008; 22: 649-655.
98. Cozzi F, Pigatto E, Rizzo M, Favaro M, Zanatta E, Cardarelli S, Riato L, Punzi L. Low occurrence of digital ulcers in scleroderma patients treated with bosentan for pulmonary arterial hypertension: a retrospective case-control study. *Clin Rheumatol* 2013; 32: 679-683.
99. Matucci-Cerinic M, Denton CP, Furst DE, Mayes MD, Hsu VM, Carpentier P, Wigley FM, Black CM, Fessler BJ, Merkel PA, Pope JE, Sweiss NJ, Doyle MK, Hellmich B, Medsger TA, Jr., Morganti A, Kramer F, Korn JH, Seibold JR. Bosentan treatment of digital ulcers related to systemic sclerosis: results from the RAPIDS-2 randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis* 2011; 70: 32-38.
100. Korn JH, Mayes M, Matucci Cerinic M, Rainisio M, Pope J, Hachulla E, Rich E, Carpentier P, Molitor J, Seibold JR, Hsu V, Guillevin L, Chatterjee S, Peter HH, Coppock J, Herrick A, Merkel PA, Simms R, Denton CP, Furst D, Nguyen N, Gaitonde M, Black C. Digital ulcers in systemic sclerosis: prevention by treatment with bosentan, an oral endothelin receptor antagonist. *Arthritis Rheum* 2004; 50: 3985-3993.
101. Cutolo M, Zampogna G, Vremis L, Smith V, Pizzorni C, Sulli A. Longterm effects of endothelin receptor antagonism on microvascular damage evaluated by nailfold capillaroscopic analysis in systemic sclerosis. *J Rheumatol* 2013; 40: 40-45.
102. Manetti M, Guiducci S, Ibba-Manneschi L, Matucci-Cerinic M. Mechanisms in the loss of capillaries in systemic sclerosis: angiogenesis versus vasculogenesis. *J Cell Mol Med* 2010; 14: 1241-1254.
103. Koch AE, Distler O. Vasculopathy and disordered angiogenesis in selected rheumatic diseases: rheumatoid arthritis and systemic sclerosis. *Arthritis Res Ther* 2007; 9 Suppl 2: S3.
104. Guiducci S, Distler O, Distler JH, Matucci-Cerinic M. Mechanisms of vascular damage in SSc--implications for vascular treatment strategies. *Rheumatology (Oxford)* 2008; 47 Suppl 5: v18-20.
105. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. *Lancet* 2004; 364: 603-610.
106. Majewski S, Skopinska-Rozewska E, Jablonska S, Polakowski I, Pawinska M, Marczak M, Szmurlo A. Modulatory effect of sera from scleroderma patients on lymphocyte-induced angiogenesis. *Arthritis Rheum* 1985; 28: 1133-1139.

107. Kahaleh MB, DeLustro F, Bock W, LeRoy EC. Human monocyte modulation of endothelial cells and fibroblast growth: possible mechanism for fibrosis. *Clin Immunol Immunopathol* 1986; 39: 242-255.
108. Marczak M, Majewski S, Skopinska-Rozewska E, Polakowski I, Jablonska S. Enhanced angiogenic capability of monocyte-enriched mononuclear cell suspensions from patients with systemic scleroderma. *J Invest Dermatol* 1986; 86: 355-358.
109. Choi JJ, Min DJ, Cho ML, Min SY, Kim SJ, Lee SS, Park KS, Seo YI, Kim WU, Park SH, Cho CS. Elevated vascular endothelial growth factor in systemic sclerosis. *J Rheumatol* 2003; 30: 1529-1533.
110. Distler O, Distler JH, Scheid A, Acker T, Hirth A, Rethage J, Michel BA, Gay RE, Muller-Ladner U, Matucci-Cerinic M, Plate KH, Gassmann M, Gay S. Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. *Circ Res* 2004; 95: 109-116.
111. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; 9: 669-676.
112. Distler O, Del Rosso A, Giacomelli R, Cipriani P, Conforti ML, Guiducci S, Gay RE, Michel BA, Bruhlmann P, Muller-Ladner U, Gay S, Matucci-Cerinic M. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002; 4: R11.
113. Mackiewicz Z, Sukura A, Povilenaite D, Ceponis A, Virtanen I, Hukkanen M, Konttinen YT. Increased but imbalanced expression of VEGF and its receptors has no positive effect on angiogenesis in systemic sclerosis skin. *Clin Exp Rheumatol* 2002; 20: 641-646.
114. Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer research* 2002; 62: 4123-4131.
115. Qiu Y, Hoareau-Aveilla C, Oltean S, Harper SJ, Bates DO. The anti-angiogenic isoforms of VEGF in health and disease. *Biochem Soc Trans* 2009; 37: 1207-1213.
116. Manetti M, Guiducci S, Romano E, Ceccarelli C, Bellando-Randone S, Conforti ML, Ibba-Manneschi L, Matucci-Cerinic M. Overexpression of VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, leads to insufficient angiogenesis in patients with systemic sclerosis. *Circ Res* 2011; 109: e14-26.
117. Manetti M, Guiducci S, Romano E, Bellando-Randone S, Lepri G, Bruni C, Conforti ML, Ibba-Manneschi L, Matucci-Cerinic M. Increased plasma levels of the VEGF165b splice variant are associated with the severity of nailfold capillary loss in systemic sclerosis. *Ann Rheum Dis* 2013; 72: 1425-1427.
118. Avouac J, Juin F, Wipff J, Couraud PO, Chiocchia G, Kahan A, Boileau C, Uzan G, Allanore Y. Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. *Ann Rheum Dis* 2008; 67: 1455-1460.
119. Allanore Y, Batteux F, Avouac J, Assous N, Weill B, Kahan A. Levels of circulating endothelial progenitor cells in systemic sclerosis. *Clin Exp Rheumatol* 2007; 25: 60-66.
120. Del Papa N, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, Maglione W, Comina DP, Vitali C, Fraticelli P, Gabrielli A, Cortelezzi A, Lambertenghi-Deliliers

G. Bone marrow endothelial progenitors are defective in systemic sclerosis. *Arthritis Rheum* 2006; 54: 2605-2615.
121. Cipriani P, Guiducci S, Miniati I, Cinelli M, Urbani S, Marrelli A, Dolo V, Pavan A, Saccardi R, Tyndall A, Giacomelli R, Cerinic MM. Impairment of endothelial cell differentiation from bone marrow-derived mesenchymal stem cells: new insight into the pathogenesis of systemic sclerosis. *Arthritis Rheum* 2007; 56: 1994-2004.
122. Duchini A, Sessoms SL. Gastrointestinal hemorrhage in patients with systemic sclerosis and CREST syndrome. *Am J Gastroenterol* 1998; 93: 1453-1456.
123. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 1995; 75: 487-517.
124. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem Pharmacol* 2009; 78: 539-552.
125. Brooke BS, Karnik SK, Li DY. Extracellular matrix in vascular morphogenesis and disease: structure versus signal. *Trends Cell Biol* 2003; 13: 51-56.
126. Gittenberger-de Groot AC, DeRuiter MC, Bergwerff M, Poelmann RE. Smooth muscle cell origin and its relation to heterogeneity in development and disease. *Arteriosclerosis, thrombosis, and vascular biology* 1999; 19: 1589-1594.
127. Frid MG, Dempsey EC, Durmowicz AG, Stenmark KR. Smooth muscle cell heterogeneity in pulmonary and systemic vessels. Importance in vascular disease. *Arteriosclerosis, thrombosis, and vascular biology* 1997; 17: 1203-1209.
128. Guiducci S, Giacomelli R, Cerinic MM. Vascular complications of scleroderma. *Autoimmun Rev* 2007; 6: 520-523.
129. Matucci-Cerinic M, Kahaleh B, Wigley FM. Review: evidence that systemic sclerosis is a vascular disease. *Arthritis Rheum* 2013; 65: 1953-1962.
130. Cerinic MM, Valentini G, Sorano GG, D'Angelo S, Cuomo G, Fenu L, Generini S, Cinotti S, Morfini M, Pignone A, Guiducci S, Del Rosso A, Kalfin R, Das D, Marongiu F. Blood coagulation, fibrinolysis, and markers of endothelial dysfunction in systemic sclerosis. *Semin Arthritis Rheum* 2003; 32: 285-295.
131. Binai N, O'Reilly S, Griffiths B, van Laar JM, Hugle T. Differentiation potential of CD14+ monocytes into myofibroblasts in patients with systemic sclerosis. *PLoS One* 2012; 7: e33508.
132. Seibold JR, Giorno RC, Claman HN. Dermal mast cell degranulation in systemic sclerosis. *Arthritis Rheum* 1990; 33: 1702-1709.
133. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; 301: 640-643.
134. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine & growth factor reviews* 2002; 13: 413-421.
135. Rocha e Silva M. A brief survey of the history of inflammation. *Agents Actions* 1978; 8: 45-49.
136. Farina A, Cirone M, York M, Lenna S, Padilla C, McLaughlin S, Faggioni A, Lafyatis R, Trojanowska M, Farina GA. Epstein-Barr virus infection induces aberrant TLR activation pathway and fibroblast-myofibroblast conversion in scleroderma. *J Invest Dermatol* 2014; 134: 954-964.
137. Tan FK, Zhou X, Mayes MD, Gourh P, Guo X, Marcum C, Jin L, Arnett FC, Jr. Signatures of differentially regulated interferon gene expression and vasculotrophism

in the peripheral blood cells of systemic sclerosis patients. *Rheumatology (Oxford)* 2006; 45: 694-702.
138. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, Brohawn P, Kiener PA, Richman L, Fiorentino D, Greenberg SA, Jallal B, Yao Y. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 2011; 70: 2029-2036.
139. Trad S, Granel B, Parizot C, Dorgham K, Hanslik T, Marie I, Amoura Z. [Cytokines and T cell differentiation in systemic sclerosis]. *Rev Med Interne* 2011; 32: 472-485.
140. Prescott RJ, Freemont AJ, Jones CJ, Hoyland J, Fielding P. Sequential dermal microvascular and perivascular changes in the development of scleroderma. *J Pathol* 1992; 166: 255-263.
141. Elisa T, Antonio P, Giuseppe P, Alessandro B, Giuseppe A, Federico C, Marzia D, Ruggero B, Giacomo M, Andrea O, Daniela R, Mariaelisa R, Claudio L. Endothelin Receptors Expressed by Immune Cells Are Involved in Modulation of Inflammation and in Fibrosis: Relevance to the Pathogenesis of Systemic Sclerosis. *J Immunol Res* 2015; 2015: 147616.
142. D'Angelo WA, Fries JF, Masi AT, Shulman LE. Pathologic observations in systemic sclerosis (scleroderma). A study of fifty-eight autopsy cases and fifty-eight matched controls. *Am J Med* 1969; 46: 428-440.
143. Fisher ER, Rodnan GP. Pathologic observations concerning the cutaneous lesion of progressive systemic sclerosis: an electron microscopic histochemical and immunohistochemical study. *Arthritis Rheum* 1960; 3: 536-545.
144. Nishioka K, Kobayashi Y, Katayama I, Takijiri C. Mast cell numbers in diffuse scleroderma. *Arch Dermatol* 1987; 123: 205-208.
145. Fleischmajer R, Perlish JS, Reeves JR. Cellular infiltrates in scleroderma skin. *Arthritis Rheum* 1977; 20: 975-984.
146. Bosello S, Angelucci C, Lama G, Alivernini S, Proietti G, Tolusso B, Sica G, Gremese E, Ferraccioli G. Characterization of inflammatory cell infiltrate of scleroderma skin: B cells and skin score progression. *Arthritis Res Ther* 2018; 20: 75.
147. Fiocco U, Rosada M, Cozzi L, Ortolani C, De Silvestro G, Ruffatti A, Cozzi E, Gallo C, Todesco S. Early phenotypic activation of circulating helper memory T cells in scleroderma: correlation with disease activity. *Ann Rheum Dis* 1993; 52: 272-277.
148. Kahan A, Kahan A, Picard F, Menkes CJ, Amor B. Abnormalities of T lymphocyte subsets in systemic sclerosis demonstrated with anti-CD45RA and anti-CD29 monoclonal antibodies. *Ann Rheum Dis* 1991; 50: 354-358.
149. Mavalia C, Scaletti C, Romagnani P, Carossino AM, Pignone A, Emmi L, Pupilli C, Pizzolo G, Maggi E, Romagnani S. Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. *Am J Pathol* 1997; 151: 1751-1758.
150. Radstake TR, van Bon L, Broen J, Hussiani A, Hesselstrand R, Wuttge DM, Deng Y, Simms R, Lubberts E, Lafyatis R. The pronounced Th17 profile in systemic sclerosis (SSc) together with intracellular expression of TGFbeta and IFNgamma distinguishes SSc phenotypes. *PLoS One* 2009; 4: e5903.
151. Yang X, Yang J, Xing X, Wan L, Li M. Increased frequency of Th17 cells in systemic sclerosis is related to disease activity and collagen overproduction. *Arthritis Res Ther* 2014; 16: R4.

152. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; 8: 942-949.
153. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 2007; 13: 108-116.
154. Radstake TR, van Bon L, Broen J, Wenink M, Santegoets K, Deng Y, Hussaini A, Simms R, Cruikshank WW, Lafyatis R. Increased frequency and compromised function of T regulatory cells in systemic sclerosis (SSc) is related to a diminished CD69 and TGFbeta expression. *PLoS One* 2009; 4: e5981.
155. Klein S, Kretz CC, Ruland V, Stumpf C, Haust M, Hartschuh W, Hartmann M, Enk A, Suri-Payer E, Oberle N, Krammer PH, Kuhn A. Reduction of regulatory T cells in skin lesions but not in peripheral blood of patients with systemic scleroderma. *Ann Rheum Dis* 2011; 70: 1475-1481.
156. Fenoglio D, Battaglia F, Parodi A, Stringara S, Negrini S, Panico N, Rizzi M, Kalli F, Conteduca G, Ghio M, De Palma R, Indiveri F, Filaci G. Alteration of Th17 and Treg cell subpopulations co-exist in patients affected with systemic sclerosis. *Clin Immunol* 2011; 139: 249-257.
157. Antiga E, Quaglino P, Bellandi S, Volpi W, Del Bianco E, Comessatti A, Osella-Abate S, De Simone C, Marzano A, Bernengo MG, Fabbri P, Caproni M. Regulatory T cells in the skin lesions and blood of patients with systemic sclerosis and morphoea. *The British journal of dermatology* 2010; 162: 1056-1063.
158. Valmori D, Raffin C, Raimbaud I, Ayyoub M. Human RORgammat+ TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-specific polarizing factors. *Proc Natl Acad Sci U S A* 2010; 107: 19402-19407.
159. Ferraccioli G, Zizzo G. The potential role of Th17 in mediating the transition from acute to chronic autoimmune inflammation: rheumatoid arthritis as a model. *Discov Med* 2011; 11: 413-424.
160. Parel Y, Aurrand-Lions M, Scheja A, Dayer JM, Roosnek E, Chizzolini C. Presence of CD4+CD8+ double-positive T cells with very high interleukin-4 production potential in lesional skin of patients with systemic sclerosis. *Arthritis Rheum* 2007; 56: 3459-3467.
161. Roumm AD, Whiteside TL, Medsger TA, Jr., Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. *Arthritis Rheum* 1984; 27: 645-653.
162. Jimenez SA. Cellular immune dysfunction and the pathogenesis of scleroderma. *Semin Arthritis Rheum* 1983; 13: 104-113.
163. van Laar JM, Farge D, Sont JK, Naraghi K, Marjanovic Z, Larghero J, Schuerwegh AJ, Marijt EW, Vonk MC, Schattenberg AV, Matucci-Cerinic M, Voskuyl AE, van de Loosdrecht AA, Daikeler T, Kotter I, Schmalzing M, Martin T, Lioure B, Weiner SM, Kreuter A, Deligny C, Durand JM, Emery P, Machold KP, Sarrot-Reynauld F, Warnatz K, Adoue DF, Constans J, Tony HP, Del Papa N, Fassas A, Himsel A, Launay D, Lo Monaco A, Philippe P, Quere I, Rich E, Westhovens R, Griffiths B, Saccardi R, van den Hoogen FH, Fibbe WE, Socie G, Gratwohl A, Tyndall A, Group EESS. Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. *JAMA* 2014; 311: 2490-2498.

164. Burt RK, Shah SJ, Dill K, Grant T, Gheorghiade M, Schroeder J, Craig R, Hirano I, Marshall K, Ruderman E, Jovanovic B, Milanetti F, Jain S, Boyce K, Morgan A, Carr J, Barr W. Autologous non-myeloablative haemopoietic stem-cell transplantation compared with pulse cyclophosphamide once per month for systemic sclerosis (ASSIST): an open-label, randomised phase 2 trial. *Lancet* 2011; 378: 498-506.
165. Taylor ML, Noble PW, White B, Wise R, Liu MC, Bochner BS. Extensive surface phenotyping of alveolar macrophages in interstitial lung disease. *Clin Immunol* 2000; 94: 33-41.
166. Ishikawa O, Ishikawa H. Macrophage infiltration in the skin of patients with systemic sclerosis. *J Rheumatol* 1992; 19: 1202-1206.
167. van Bon L, Popa C, Huijbens R, Vonk M, York M, Simms R, Hesselstrand R, Wuttge DM, Lafyatis R, Radstake TR. Distinct evolution of TLR-mediated dendritic cell cytokine secretion in patients with limited and diffuse cutaneous systemic sclerosis. *Ann Rheum Dis* 2010; 69: 1539-1547.
168. Schwartz DM, Bonelli M, Gadina M, O'Shea JJ. Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. *Nat Rev Rheumatol* 2016; 12: 25-36.
169. Dinarello CA. Historical insights into cytokines. *Eur J Immunol* 2007; 37 Suppl 1: S34-45.
170. Raja J, Denton CP. Cytokines in the immunopathology of systemic sclerosis. *Semin Immunopathol* 2015; 37: 543-557.
171. Hasegawa M, Sato S, Fujimoto M, Ihn H, Kikuchi K, Takehara K. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *J Rheumatol* 1998; 25: 308-313.
172. Barnes TC, Anderson ME, Moots RJ. The many faces of interleukin-6: the role of IL-6 in inflammation, vasculopathy, and fibrosis in systemic sclerosis. *Int J Rheumatol* 2011; 2011: 721608.
173. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 1986; 324: 73-76.
174. Shima Y, Kuwahara Y, Murota H, Kitaba S, Kawai M, Hirano T, Arimitsu J, Narazaki M, Hagihara K, Ogata A, Katayama I, Kawase I, Kishimoto T, Tanaka T. The skin of patients with systemic sclerosis softened during the treatment with anti-IL-6 receptor antibody tocilizumab. *Rheumatology (Oxford)* 2010; 49: 2408-2412.
175. Kawaguchi Y, Hara M, Kamatani N, Wright TM. Identification of an IL1A gene segment that determines aberrant constitutive expression of interleukin-1 alpha in systemic sclerosis. *Arthritis Rheum* 2003; 48: 193-202.
176. Kawaguchi Y. Role of the interleukin-1 family in the fibrogenic phenotype in systemic sclerosis. In: Takehara K, Fujimoto M, Kuwana M, editors. Systemic Sclerosis - Basic and Translational Research. Tokyo: Springer; 2016. p. 103-116.
177. Kawaguchi Y, Tochimoto A, Ichikawa N, Harigai M, Hara M, Kotake S, Kitamura Y, Kamatani N. Association of IL1A gene polymorphisms with susceptibility to and severity of systemic sclerosis in the Japanese population. *Arthritis Rheum* 2003; 48: 186-192.
178. Berger A. Th1 and Th2 responses: what are they? *BMJ* 2000; 321: 424.

179. Streicher K, Morehouse CA, Groves CJ, Rajan B, Pilataxi F, Lehmann KP, Brohawn PZ, Higgs BW, McKeever K, Greenberg SA, Fiorentino D, Richman LK, Jallal B, Herbst R, Yao Y, Ranade K. The plasma cell signature in autoimmune disease. *Arthritis Rheumatol* 2014; 66: 173-184.
180. Wu M, Mohan C. B-cells in systemic sclerosis: emerging evidence from genetics to phenotypes. *Curr Opin Rheumatol* 2015; 27: 537-541.
181. Davidson A, Diamond B. Autoimmune diseases. *N Engl J Med* 2001; 345: 340-350.
182. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, Distler O, group ERs. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. *Ann Rheum Dis* 2015; 74: 1188-1194.
183. Francois A, Chatelus E, Wachsmann D, Sibilia J, Bahram S, Alsaleh G, Gottenberg JE. B lymphocytes and B-cell activating factor promote collagen and profibrotic markers expression by dermal fibroblasts in systemic sclerosis. *Arthritis Res Ther* 2013; 15: R168.
184. Jones SA, Richards PJ, Scheller J, Rose-John S. IL-6 transsignaling: the in vivo consequences. *J Interferon Cytokine Res* 2005; 25: 241-253.
185. Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. *J Immunol* 2000; 165: 6635-6643.
186. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis Rheum* 2004; 50: 1918-1927.
187. Hasegawa M, Fujimoto M, Takehara K, Sato S. Pathogenesis of systemic sclerosis: altered B cell function is the key linking systemic autoimmunity and tissue fibrosis. *J Dermatol Sci* 2005; 39: 1-7.
188. Shovman O, Gilburd B, Barzilai O, Shinar E, Larida B, Zandman-Goddard G, Binder SR, Shoenfeld Y. Evaluation of the BioPlex 2200 ANA screen: analysis of 510 healthy subjects: incidence of natural/predictive autoantibodies. *Ann N Y Acad Sci* 2005; 1050: 380-388.
189. Mehra S, Walker J, Patterson K, Fritzler MJ. Autoantibodies in systemic sclerosis. *Autoimmun Rev* 2013; 12: 340-354.
190. Smeenk RJ. Antinuclear antibodies: cause of disease or caused by disease? *Rheumatology (Oxford)* 2000; 39: 581-584.
191. Utz PJ, Anderson P. Posttranslational protein modifications, apoptosis, and the bypass of tolerance to autoantigens. *Arthritis Rheum* 1998; 41: 1152-1160.
192. Schachna L, Wigley FM, Morris S, Gelber AC, Rosen A, Casciola-Rosen L. Recognition of Granzyme B-generated autoantigen fragments in scleroderma patients with ischemic digital loss. *Arthritis Rheum* 2002; 46: 1873-1884.
193. Zhou X, Tan FK, Xiong M, Milewicz DM, Feghali CA, Fritzler MJ, Reveille JD, Arnett FC. Systemic sclerosis (scleroderma): specific autoantigen genes are selectively overexpressed in scleroderma fibroblasts. *J Immunol* 2001; 167: 7126-7133.
194. Lafyatis R, York M. Innate immunity and inflammation in systemic sclerosis. *Curr Opin Rheumatol* 2009; 21: 617-622.
195. Svegliati S, Olivieri A, Campelli N, Luchetti M, Poloni A, Trappolini S, Moroncini G, Bacigalupo A, Leoni P, Avvedimento EV, Gabrielli A. Stimulatory autoantibodies to

PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood* 2007; 110: 237-241.
196. Kurasawa K, Arai S, Owada T, Maezawa R, Kumano K, Fukuda T. Autoantibodies against platelet-derived growth factor receptor alpha in patients with systemic lupus erythematosus. *Mod Rheumatol* 2010; 20: 458-465.
197. Loizos N, Lariccia L, Weiner J, Griffith H, Boin F, Hummers L, Wigley F, Kussie P. Lack of detection of agonist activity by antibodies to platelet-derived growth factor receptor alpha in a subset of normal and systemic sclerosis patient sera. *Arthritis Rheum* 2009; 60: 1145-1151.
198. Balada E, Simeon-Aznar CP, Ordi-Ros J, Rosa-Leyva M, Selva-O'Callaghan A, Pardos-Gea J, Fonollosa-Pla V, Vilardell-Tarres M. Anti-PDGFR-alpha antibodies measured by non-bioactivity assays are not specific for systemic sclerosis. *Ann Rheum Dis* 2008; 67: 1027-1029.
199. Classen JF, Henrohn D, Rorsman F, Lennartsson J, Lauwerys BR, Wikstrom G, Rorsman C, Lenglez S, Franck-Larsson K, Tomasi JP, Kampe O, Vanthuyne M, Houssiau FA, Demoulin JB. Lack of evidence of stimulatory autoantibodies to platelet-derived growth factor receptor in patients with systemic sclerosis. *Arthritis Rheum* 2009; 60: 1137-1144.
200. Gabrielli A, Moroncini G, Svegliati S, Avvedimento EV. Autoantibodies against the platelet-derived growth factor receptor in scleroderma: comment on the articles by Classen et al and Loizos et al. *Arthritis Rheum* 2009; 60: 3521-3522.
201. Kuwana M, Medsger TA. The clinical aspects of autoantibodies. In: Varga J, Denton C, Wigley FM, Allanore Y, Kuwana M, editors. Scleroderma - From Pathogenesis to Comprehensive Management. New York: Springer; 2017. p. 207-220.
202. Miyawaki S, Asanuma H, Nishiyama S, Yoshinaga Y. Clinical and serological heterogeneity in patients with anticentromere antibodies. *J Rheumatol* 2005; 32: 1488-1494.
203. Reveille JD, Solomon DH, American College of Rheumatology Ad Hoc Committee of Immunologic Testing G. Evidence-based guidelines for the use of immunologic tests: anticentromere, Scl-70, and nucleolar antibodies. *Arthritis Rheum* 2003; 49: 399-412.
204. Van Eenennaam H, Vogelzangs JH, Bisschops L, Te Boome LC, Seelig HP, Renz M, De Rooij DJ, Brouwer R, Pluk H, Pruijn GJ, Van Venrooij WJ, Van Den Hoogen FH. Autoantibodies against small nucleolar ribonucleoprotein complexes and their clinical associations. *Clinical and experimental immunology* 2002; 130: 532-540.
205. Kuwana M, Kimura K, Hirakata M, Kawakami Y, Ikeda Y. Differences in autoantibody response to Th/To between systemic sclerosis and other autoimmune diseases. *Ann Rheum Dis* 2002; 61: 842-846.
206. Mitri GM, Lucas M, Fertig N, Steen VD, Medsger TA, Jr. A comparison between anti-Th/To- and anticentromere antibody-positive systemic sclerosis patients with limited cutaneous involvement. *Arthritis Rheum* 2003; 48: 203-209.
207. Okano Y, Medsger TA, Jr. Autoantibody to Th ribonucleoprotein (nucleolar 7-2 RNA protein particle) in patients with systemic sclerosis. *Arthritis Rheum* 1990; 33: 1822-1828.
208. Satoh M, Kuwana M, Ogasawara T, Ajmani AK, Langdon JJ, Kimpel D, Wang J, Reeves WH. Association of autoantibodies to topoisomerase I and the phosphorylated (IIO)

form of RNA polymerase II in Japanese scleroderma patients. *J Immunol* 1994; 153: 5838-5848.
209. Kuwana M, Kimura K, Kawakami Y. Identification of an immunodominant epitope on RNA polymerase III recognized by systemic sclerosis sera: application to enzyme-linked immunosorbent assay. *Arthritis Rheum* 2002; 46: 2742-2747.
210. Harvey GR, Butts S, Rands AL, Patel Y, McHugh NJ. Clinical and serological associations with anti-RNA polymerase antibodies in systemic sclerosis. *Clinical and experimental immunology* 1999; 117: 395-402.
211. Brouwer R, Pruijn GJ, van Venrooij WJ. The human exosome: an autoantigenic complex of exoribonucleases in myositis and scleroderma. *Arthritis Res* 2001; 3: 102-106.
212. Brouwer R, Vree Egberts WT, Hengstman GJ, Raijmakers R, van Engelen BG, Seelig HP, Renz M, Mierau R, Genth E, Pruijn GJ, van Venrooij WJ. Autoantibodies directed to novel components of the PM/Scl complex, the human exosome. *Arthritis Res* 2002; 4: 134-138.
213. Yang JM, Baserga SJ, Turley SJ, Pollard KM. Fibrillarin and other snoRNP proteins are targets of autoantibodies in xenobiotic-induced autoimmunity. *Clin Immunol* 2001; 101: 38-50.
214. Arnett FC, Reveille JD, Goldstein R, Pollard KM, Leaird K, Smith EA, Leroy EC, Fritzler MJ. Autoantibodies to fibrillarin in systemic sclerosis (scleroderma). An immunogenetic, serologic, and clinical analysis. *Arthritis Rheum* 1996; 39: 1151-1160.
215. Steen V, Medsger TA, Jr. Predictors of isolated pulmonary hypertension in patients with systemic sclerosis and limited cutaneous involvement. *Arthritis Rheum* 2003; 48: 516-522.
216. Worda M, Sgonc R, Dietrich H, Niederegger H, Sundick RS, Gershwin ME, Wick G. In vivo analysis of the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay. *Arthritis Rheum* 2003; 48: 2605-2614.
217. Nishijima C, Hayakawa I, Matsushita T, Komura K, Hasegawa M, Takehara K, Sato S. Autoantibody against matrix metalloproteinase-3 in patients with systemic sclerosis. *Clinical and experimental immunology* 2004; 138: 357-363.
218. Sato S, Hayakawa I, Hasegawa M, Fujimoto M, Takehara K. Function blocking autoantibodies against matrix metalloproteinase-1 in patients with systemic sclerosis. *J Invest Dermatol* 2003; 120: 542-547.
219. Kawaguchi Y, Nakamura Y, Matsumoto I, Nishimagi E, Satoh T, Kuwana M, Sumida T, Hara M. Muscarinic-3 acetylcholine receptor autoantibody in patients with systemic sclerosis: contribution to severe gastrointestinal tract dysmotility. *Ann Rheum Dis* 2009; 68: 710-714.
220. Riemekasten G, Philippe A, Nather M, Slowinski T, Muller DN, Heidecke H, Matucci-Cerinic M, Czirjak L, Lukitsch I, Becker M, Kill A, van Laar JM, Catar R, Luft FC, Burmester GR, Hegner B, Dragun D. Involvement of functional autoantibodies against vascular receptors in systemic sclerosis. *Ann Rheum Dis* 2011; 70: 530-536.
221. Tan FK, Arnett FC, Antohi S, Saito S, Mirarchi A, Spiera H, Sasaki T, Shoichi O, Takeuchi K, Pandey JP, Silver RM, LeRoy C, Postlethwaite AE, Bona CA. Autoantibodies to the extracellular matrix microfibrillar protein, fibrillin-1, in patients with scleroderma and other connective tissue diseases. *J Immunol* 1999; 163: 1066-1072.

222. Gantwerker EA, Hom DB. Skin: histology and physiology of wound healing. *Facial Plast Surg Clin North Am* 2011; 19: 441-453.
223. Generini S, Fiori G, Moggi Pignone A, Matucci Cerinic M, Cagnoni M. Systemic sclerosis. A clinical overview. *Adv Exp Med Biol* 1999; 455: 73-83.
224. Lambova S. Cardiac manifestations in systemic sclerosis. *World J Cardiol* 2014; 6: 993-1005.
225. Tzelepis GE, Kelekis NL, Plastiras SC, Mitseas P, Economopoulos N, Kampolis C, Gialafos EJ, Moyssakis I, Moutsopoulos HM. Pattern and distribution of myocardial fibrosis in systemic sclerosis: a delayed enhanced magnetic resonance imaging study. *Arthritis Rheum* 2007; 56: 3827-3836.
226. Cappelli S, Bellando Randone S, Camiciottoli G, De Paulis A, Guiducci S, Matucci-Cerinic M. Interstitial lung disease in systemic sclerosis: where do we stand? *Eur Respir Rev* 2015; 24: 411-419.
227. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. *Nat Rev Rheumatol* 2011; 8: 42-54.
228. Strehlow D, Korn JH. Biology of the scleroderma fibroblast. *Curr Opin Rheumatol* 1998; 10: 572-578.
229. Kisseleva T, Brenner DA. Mechanisms of fibrogenesis. *Exp Biol Med (Maywood)* 2008; 233: 109-122.
230. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003; 200: 500-503.
231. Shephard P, Hinz B, Smola-Hess S, Meister JJ, Krieg T, Smola H. Dissecting the roles of endothelin, TGF-beta and GM-CSF on myofibroblast differentiation by keratinocytes. *Thromb Haemost* 2004; 92: 262-274.
232. LeRoy EC, Trojanowska MI, Smith EA. Cytokines and human fibrosis. *Eur Cytokine Netw* 1990; 1: 215-219.
233. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 1999; 277: C1-9.
234. Ho YY, Lagares D, Tager AM, Kapoor M. Fibrosis--a lethal component of systemic sclerosis. *Nat Rev Rheumatol* 2014; 10: 390-402.
235. Kendall RT, Feghali-Bostwick CA. Fibroblasts in fibrosis: novel roles and mediators. *Front Pharmacol* 2014; 5: 123.
236. Varga JA, Trojanowska M. Fibrosis in systemic sclerosis. *Rheum Dis Clin North Am* 2008; 34: 115-143; vii.
237. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB J* 2004; 18: 816-827.
238. Kirk TZ, Mark ME, Chua CC, Chua BH, Mayes MD. Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. *J Biol Chem* 1995; 270: 3423-3428.
239. Kikuchi K, Kubo M, Sato S, Fujimoto M, Tamaki K. Serum tissue inhibitor of metalloproteinases in patients with systemic sclerosis. *J Am Acad Dermatol* 1995; 33: 973-978.
240. Rinkevich Y, Walmsley GG, Hu MS, Maan ZN, Newman AM, Drukker M, Januszyk M, Krampitz GW, Gurtner GC, Lorenz HP, Weissman IL, Longaker MT. Skin fibrosis. Identification and isolation of a dermal lineage with intrinsic fibrogenic potential. *Science* 2015; 348: aaa2151.

241. Allt G, Lawrenson JG. Pericytes: cell biology and pathology. *Cells Tissues Organs* 2001; 169: 1-11.
242. Shepro D, Morel NM. Pericyte physiology. *FASEB J* 1993; 7: 1031-1038.
243. Carmeliet P. Blood vessels and nerves: common signals, pathways and diseases. *Nat Rev Genet* 2003; 4: 710-720.
244. Rajkumar VS, Howell K, Csiszar K, Denton CP, Black CM, Abraham DJ. Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. *Arthritis Res Ther* 2005; 7: R1113-1123.
245. Asano Y, Stawski L, Hant F, Highland K, Silver R, Szalai G, Watson DK, Trojanowska M. Endothelial Fli1 deficiency impairs vascular homeostasis: a role in scleroderma vasculopathy. *Am J Pathol* 2010; 176: 1983-1998.
246. Rajkumar VS, Sundberg C, Abraham DJ, Rubin K, Black CM. Activation of microvascular pericytes in autoimmune Raynaud's phenomenon and systemic sclerosis. *Arthritis Rheum* 1999; 42: 930-941.
247. Li Z, Jimenez SA. Protein kinase Cdelta and c-Abl kinase are required for transforming growth factor beta induction of endothelial-mesenchymal transition in vitro. *Arthritis Rheum* 2011; 63: 2473-2483.
248. Manetti M, Guiducci S, Matucci-Cerinic M. The origin of the myofibroblast in fibroproliferative vasculopathy: does the endothelial cell steer the pathophysiology of systemic sclerosis? *Arthritis Rheum* 2011; 63: 2164-2167.
249. Jimenez SA. Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. *ISRN Rheumatol* 2013; 2013: 835948.
250. Lawrence DA. Transforming growth factor-beta: a general review. *Eur Cytokine Netw* 1996; 7: 363-374.
251. Sporn MB, Roberts AB, Wakefield LM, Assoian RK. Transforming growth factor-beta: biological function and chemical structure. *Science* 1986; 233: 532-534.
252. Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. *J Dermatol Sci* 2008; 49: 103-113.
253. Biernacka A, Dobaczewski M, Frangogiannis NG. TGF-beta signaling in fibrosis. *Growth Factors* 2011; 29: 196-202.
254. Kulozik M, Hogg A, Lankat-Buttgereit B, Krieg T. Co-localization of transforming growth factor beta 2 with alpha 1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. *J Clin Invest* 1990; 86: 917-922.
255. Oi M, Yamamoto T, Nishioka K. Increased expression of TGF-beta1 in the sclerotic skin in bleomycin-'susceptible' mouse strains. *J Med Dent Sci* 2004; 51: 7-17.
256. Varga J, Pasche B. Transforming growth factor beta as a therapeutic target in systemic sclerosis. *Nat Rev Rheumatol* 2009; 5: 200-206.
257. Gordon J, Udeh U, Doobay K, Magro C, Wildman H, Davids M, Mersten JN, Huang WT, Lyman S, Crow MK, Spiera RF. Imatinib mesylate (Gleevec) in the treatment of diffuse cutaneous systemic sclerosis: results of a 24-month open label, extension phase, single-centre trial. *Clin Exp Rheumatol* 2014; 32: S-189-193.
258. Akhmetshina A, Venalis P, Dees C, Busch N, Zwerina J, Schett G, Distler O, Distler JH. Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis. *Arthritis Rheum* 2009; 60: 219-224.

259. Distler JH, Jungel A, Huber LC, Schulze-Horsel U, Zwerina J, Gay RE, Michel BA, Hauser T, Schett G, Gay S, Distler O. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. *Arthritis Rheum* 2007; 56: 311-322.
260. Codullo V, Breda S, Meloni F, Cova E, Inghilleri S, Colombo M, Prosperi D, Malatesta M, Calderan L, Montecucco C, Distler J. SAT0315 Imatinib-loaded targeted gold nanoparticles ameliorate experimental lung fibrosis induced by bleomycin. *Annals of the Rheumatic Diseases* 2017; 76: 891-891.
261. Furst D. SP0012 Novel therapies that may make it into the clinic in systemic sclerosis. *Annals of the Rheumatic Diseases* 2018; 77: 4-4.
262. Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine & growth factor reviews* 2004; 15: 197-204.
263. Malarkey K, Belham CM, Paul A, Graham A, McLees A, Scott PH, Plevin R. The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. *Biochem J* 1995; 309 (Pt 2): 361-375.
264. Betsholtz C. Biology of platelet-derived growth factors in development. *Birth Defects Res C Embryo Today* 2003; 69: 272-285.
265. Miyazawa K, Backstrom G, Leppanen O, Persson C, Wernstedt C, Hellman U, Heldin CH, Ostman A. Role of immunoglobulin-like domains 2-4 of the platelet-derived growth factor alpha-receptor in ligand-receptor complex assembly. *J Biol Chem* 1998; 273: 25495-25502.
266. Wiesmann C, Muller YA, de Vos AM. Ligand-binding sites in Ig-like domains of receptor tyrosine kinases. *J Mol Med* 2000; 78: 247-260.
267. Black PC, Brown GA, Dinney CP, Kassouf W, Inamoto T, Arora A, Gallagher D, Munsell MF, Bar-Eli M, McConkey DJ, Adam L. Receptor heterodimerization: a new mechanism for platelet-derived growth factor induced resistance to anti-epidermal growth factor receptor therapy for bladder cancer. *The Journal of urology* 2011; 185: 693-700.
268. Saito Y, Haendeler J, Hojo Y, Yamamoto K, Berk BC. Receptor heterodimerization: essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. *Mol Cell Biol* 2001; 21: 6387-6394.
269. Klareskog L, Gustafsson R, Scheynius A, Hallgren R. Increased expression of platelet-derived growth factor type B receptors in the skin of patients with systemic sclerosis. *Arthritis Rheum* 1990; 33: 1534-1541.
270. Gay S, Jones RE, Jr., Huang GQ, Gay RE. Immunohistologic demonstration of platelet-derived growth factor (PDGF) and sis-oncogene expression in scleroderma. *J Invest Dermatol* 1989; 92: 301-303.
271. Yamakage A, Kikuchi K, Smith EA, LeRoy EC, Trojanowska M. Selective upregulation of platelet-derived growth factor alpha receptors by transforming growth factor beta in scleroderma fibroblasts. *J Exp Med* 1992; 175: 1227-1234.
272. Ludwicka A, Ohba T, Trojanowska M, Yamakage A, Strange C, Smith EA, Leroy EC, Sutherland S, Silver RM. Elevated levels of platelet derived growth factor and transforming growth factor-beta 1 in bronchoalveolar lavage fluid from patients with scleroderma. *J Rheumatol* 1995; 22: 1876-1883.
273. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1alpha from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. *J Clin Invest* 1999; 103: 1253-1260.

274. Svegliati S, Cancello R, Sambo P, Luchetti M, Paroncini P, Orlandini G, Discepoli G, Paterno R, Santillo M, Cuozzo C, Cassano S, Avvedimento EV, Gabrielli A. Platelet-derived growth factor and reactive oxygen species (ROS) regulate Ras protein levels in primary human fibroblasts via ERK1/2. Amplification of ROS and Ras in systemic sclerosis fibroblasts. *J Biol Chem* 2005; 280: 36474-36482.
275. Akhmetshina A, Dees C, Pileckyte M, Maurer B, Axmann R, Jungel A, Zwerina J, Gay S, Schett G, Distler O, Distler JH. Dual inhibition of c-abl and PDGF receptor signaling by dasatinib and nilotinib for the treatment of dermal fibrosis. *FASEB J* 2008; 22: 2214-2222.
276. Kavian N, Servettaz A, Marut W, Nicco C, Chereau C, Weill B, Batteux F. Sunitinib inhibits the phosphorylation of platelet-derived growth factor receptor beta in the skin of mice with scleroderma-like features and prevents the development of the disease. *Arthritis Rheum* 2012; 64: 1990-2000.
277. Zymek P, Nah DY, Bujak M, Ren G, Koerting A, Leucker T, Huebener P, Taffet G, Entman M, Frangogiannis NG. Interleukin-10 is not a critical regulator of infarct healing and left ventricular remodeling. *Cardiovasc Res* 2007; 74: 313-322.
278. Liao CH, Akazawa H, Tamagawa M, Ito K, Yasuda N, Kudo Y, Yamamoto R, Ozasa Y, Fujimoto M, Wang P, Nakauchi H, Nakaya H, Komuro I. Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts. *J Clin Invest* 2010; 120: 242-253.
279. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000; 19: 3159-3167.
280. Schneider MR, Wolf E. The epidermal growth factor receptor ligands at a glance. *J Cell Physiol* 2009; 218: 460-466.
281. Scaltriti M, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin Cancer Res* 2006; 12: 5268-5272.
282. Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res* 2003; 284: 54-65.
283. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* 1997; 16: 1647-1655.
284. Tokiyama K, Yokota E, Niho Y. Epidermal growth factor receptor of fibroblasts from patients with scleroderma. *J Rheumatol* 1990; 17: 1463-1468.
285. Planque S, Zhou YX, Nishiyama Y, Sinha M, O'Connor-Mccourt M, Arnett FC, Paul S. Autoantibodies to the epidermal growth factor receptor in systemic sclerosis, lupus, and autoimmune mice. *FASEB J* 2003; 17: 136-143.
286. Smith GP, Chan ES. Molecular pathogenesis of skin fibrosis: insight from animal models. *Curr Rheumatol Rep* 2010; 12: 26-33.
287. Christner PJ, Jimenez SA. Animal models of systemic sclerosis: insights into systemic sclerosis pathogenesis and potential therapeutic approaches. *Curr Opin Rheumatol* 2004; 16: 746-752.
288. Asano Y, Distler J. Overview of animal models. In: Varga J, Denton C, Wigley FM, Allanore Y, Kuwana M, editors. Scleroderma - From Pathogenesis to Comprehensive Management. New York: Springer; 2017. p. 281-293.

289. Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, Shinkai H, Nishioka K. Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *J Invest Dermatol* 1999; 112: 456-462.
290. Maurer B, Distler JH, Distler O. The Fra-2 transgenic mouse model of systemic sclerosis. *Vascular pharmacology* 2013; 58: 194-201.
291. Eferl R, Hasselblatt P, Rath M, Popper H, Zenz R, Komnenovic V, Idarraga MH, Kenner L, Wagner EF. Development of pulmonary fibrosis through a pathway involving the transcription factor Fra-2/AP-1. *Proc Natl Acad Sci U S A* 2008; 105: 10525-10530.
292. Reich N, Maurer B, Akhmetshina A, Venalis P, Dees C, Zerr P, Palumbo K, Zwerina J, Nevskaya T, Gay S, Distler O, Schett G, Distler JH. The transcription factor Fra-2 regulates the production of extracellular matrix in systemic sclerosis. *Arthritis Rheum* 2010; 62: 280-290.
293. Haynes DC, Gershwin ME. Diversity of autoantibodies in avian scleroderma. An inherited fibrotic disease of White Leghorn chickens. *J Clin Invest* 1984; 73: 1557-1568.
294. Wilson TJ, Van de Water J, Mohr FC, Boyd RL, Ansari A, Wick G, Gershwin ME. Avian scleroderma: evidence for qualitative and quantitative T cell defects. *J Autoimmun* 1992; 5: 261-276.
295. Gershwin ME, Abplanalp H, Castles JJ, Ikeda RM, van der Water J, Eklund J, Haynes D. Characterization of a spontaneous disease of white leghorn chickens resembling progressive systemic sclerosis (scleroderma). *J Exp Med* 1981; 153: 1640-1659.
296. Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest* 1996; 98: 785-792.
297. Noda S, Asano Y, Nishimura S, Taniguchi T, Fujiu K, Manabe I, Nakamura K, Yamashita T, Saigusa R, Akamata K, Takahashi T, Ichimura Y, Toyama T, Tsuruta D, Trojanowska M, Nagai R, Sato S. Simultaneous downregulation of KLF5 and Fli1 is a key feature underlying systemic sclerosis. *Nat Commun* 2014; 5: 5797.
298. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, McCalmont TH, Brown PO, Botstein D, Connolly MK. Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci U S A* 2003; 100: 12319-12324.
299. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis Rheum* 2006; 54: 2271-2279.
300. Moroncini G, Grieco A, Nacci G, Paolini C, Tonnini C, Pozniak KN, Cuccioloni M, Mozzicafreddo M, Svegliati S, Angeletti M, Kazlauskas A, Avvedimento EV, Funaro A, Gabrielli A. Epitope Specificity Determines Pathogenicity and Detectability of Anti-Platelet-Derived Growth Factor Receptor alpha Autoantibodies in Systemic Sclerosis. *Arthritis Rheumatol* 2015; 67: 1891-1903.
301. Svegliati S, Amico D, Spadoni T, Fischetti C, Finke D, Moroncini G, Paolini C, Tonnini C, Grieco A, Rovinelli M, Gabrielli A. Agonistic Anti-PDGF Receptor Autoantibodies from Patients with Systemic Sclerosis Impact Human Pulmonary Artery Smooth Muscle Cells Function In Vitro. *Front Immunol* 2017; 8: 75.
302. Blobel CP. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* 2005; 6: 32-43.

303. Bhola NE, Grandis JR. Crosstalk between G-protein-coupled receptors and epidermal growth factor receptor in cancer. *Front Biosci* 2008; 13: 1857-1865.
304. Kalmes A, Daum G, Clowes AW. EGFR transactivation in the regulation of SMC function. *Ann N Y Acad Sci* 2001; 947: 42-54; discussion 54-45.
305. Carpenter G. EGF receptor transactivation mediated by the proteolytic production of EGF-like agonists. *Sci STKE* 2000; 2000: pe1.
306. Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* 2001; 20: 1594-1600.
307. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 1999; 402: 884-888.
308. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 1996; 379: 557-560.
309. Bokemeyer D, Schmitz U, Kramer HJ. Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. *Kidney Int* 2000; 58: 549-558.
310. Amorino GP, Deeble PD, Parsons SJ. Neurotensin stimulates mitogenesis of prostate cancer cells through a novel c-Src/Stat5b pathway. *Oncogene* 2007; 26: 745-756.
311. Roudabush FL, Pierce KL, Maudsley S, Khan KD, Luttrell LM. Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. *J Biol Chem* 2000; 275: 22583-22589.
312. Lee CW, Lin CC, Lin WN, Liang KC, Luo SF, Wu CB, Wang SW, Yang CM. TNF-alpha induces MMP-9 expression via activation of Src/EGFR, PDGFR/PI3K/Akt cascade and promotion of NF-kappaB/p300 binding in human tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: L799-812.
313. Decker SJ, Harris P. Effects of platelet-derived growth factor on phosphorylation of the epidermal growth factor receptor in human skin fibroblasts. *J Biol Chem* 1989; 264: 9204-9209.
314. Countaway JL, Girones N, Davis RJ. Reconstitution of epidermal growth factor receptor transmodulation by platelet-derived growth factor in Chinese hamster ovary cells. *J Biol Chem* 1989; 264: 13642-13647.
315. Schlessinger J. The epidermal growth factor receptor as a multifunctional allosteric protein. *Biochemistry* 1988; 27: 3119-3123.
316. Liu P, Ying Y, Ko YG, Anderson RG. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J Biol Chem* 1996; 271: 10299-10303.
317. Mineo C, James GL, Smart EJ, Anderson RG. Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *J Biol Chem* 1996; 271: 11930-11935.
318. Habib AA, Hognason T, Ren J, Stefansson K, Ratan RR. The epidermal growth factor receptor associates with and recruits phosphatidylinositol 3-kinase to the platelet-derived growth factor beta receptor. *J Biol Chem* 1998; 273: 6885-6891.
319. Walker F, deBlaquiere J, Burgess AW. Translocation of pp60c-src from the plasma membrane to the cytosol after stimulation by platelet-derived growth factor. *J Biol Chem* 1993; 268: 19552-19558.

320. Walker F, Burgess AW. Reconstitution of the high affinity epidermal growth factor receptor on cell-free membranes after transmodulation by platelet-derived growth factor. *J Biol Chem* 1991; 266: 2746-2752.
321. Li J, Kim YN, Bertics PJ. Platelet-derived growth factor-stimulated migration of murine fibroblasts is associated with epidermal growth factor receptor expression and tyrosine phosphorylation. *J Biol Chem* 2000; 275: 2951-2958.
322. He H, Levitzki A, Zhu HJ, Walker F, Burgess A, Maruta H. Platelet-derived growth factor requires epidermal growth factor receptor to activate p21-activated kinase family kinases. *J Biol Chem* 2001; 276: 26741-26744.
323. Donovan J, Shiwen X, Norman J, Abraham D. Platelet-derived growth factor alpha and beta receptors have overlapping functional activities towards fibroblasts. *Fibrogenesis Tissue Repair* 2013; 6: 10.
324. Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 1999; 126: 3047-3055.